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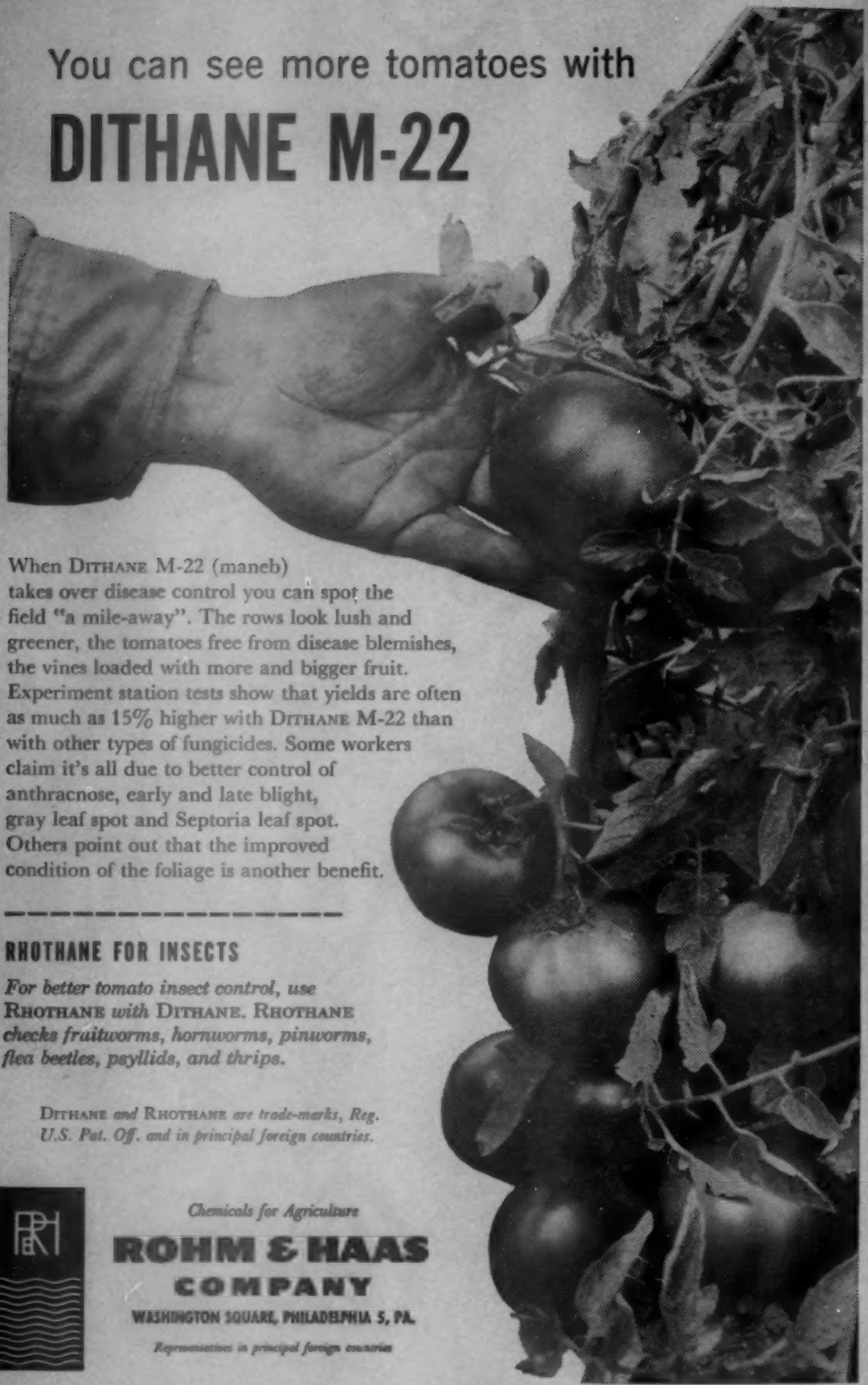

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INHERITANCE OF RESISTANCE TO ROOT-KNOT NEMATODES IN PEPPER [1]

W. W. Hare [2]

SUMMARY

The evidence from F_1, F_2, and F_3 generations of crosses of Santanka xS, a pepper resistant to *Meloidogyne incognita*, with 3 commercial bell varieties and from F_1, F_2, F_3, and backcross generations of a second resistant pepper, 405B Mexico, crossed with 2 commercial bell varieties, fitted the hypothesis that resistance is controlled by a single gene. Resistance was dominant to susceptibility. The same gene or a very closely linked gene also controlled resistance to *M. incognita acrita*. Behavior in both sets of crosses was similar, suggesting that the same gene pair was involved. The symbols *N* and *n* are suggested for this gene pair.

All of the parents were resistant to *M. arenaria* and *M. javanica*. The described gene also may have controlled resistance to these nematodes, but no definite conclusions could be drawn from the data. All of the parents were susceptible to *M. hapla*.

It has been shown (5) that pepper varieties (*Capsicum frutescens* L.) differ in reaction to the root-knot nematode *Meloidogyne incognita acrita* Chitwood. Later (6), 7 selected varieties were compared in reaction to 5 root-knot nematodes: *M. incognita* (Kofoid & White) Chitwood, *M. incognita acrita*, *M. javanica* (Treub) Chitwood, *M. arenaria* (Neal) Chitwood, and *M. hapla* Chitwood. Two varieties, 405B Mexico and Santanka xS, were resistant to all the nematode species except *M. hapla*. Commercial bell varieties were also susceptible to *M. hapla*, generally resistant to *M. arenaria* and *M. javanica*, but susceptible to *M. incognita* and *M. incognita acrita*.

Barrons (1) worked with resistance to root knot in garden bean and concluded that resistance is controlled by 2 recessive genes. Weinberger et al. (9) found that resistance to root knot was completely dominant in certain peach crosses, but they did not go beyond the F_1 generation. Gilbert and McGuire (3) did not reach final conclusions for resistance in tomato, but they postulated that resistance is dominant and controlled by 1 or 2 genes with the possibility of modifiers. Similarly, Lider's results with *Vitis* spp. (7) were not conclusive. Of the investigators mentioned above, Lider was the only one that used root-knot nematodes classified according to Chitwood's revision of the genus *Meloidogyne* (2).

With nematodes other than those causing root knot, Nilsson-Ehle (8) discovered resistance in barley to *Heterodera schachtii* Schmidt (probably *H. avenae* (Mortensen, Rostrup, & Kølpin Rayn) Filip.) that was dominant in the F_1 from a cross of resistant × susceptible. He concluded that resistance was controlled by a single dominant gene but indicated that he had not obtained adequate experimental proof and gave the reasons for the failure.

The present report concerns investigation of the inheritance of the differential resistance to root-knot nematodes in pepper. A brief report was made in 1956 (4). In 1957, Winstead and Barham (10) reported single-gene control of resistance to root knot in tomato.

METHODS AND MATERIALS.—Three commercial varieties of pepper, Ruby King, California Wonder Special, and Truheart, previously determined susceptible to *M. incognita*, were crossed to a resistant variety, Santanka xS, and the commercial varieties Burlington and Truheart were crossed to another resistant variety, 405B Mexico. The F_1, F_2, and F_3 generations from the 5 crosses were tested for resistance to *M. incognita*[3] by growing them in soil infested with nematodes. Techniques of inoculation, growing of the plants, rating of plants into disease classes, preparation of indices, etc., were the same as described earlier (5, 6).

Space requirements prohibited testing all progenies at the same time. A series of 9 tests was conducted. Plans were to use only *M. incognita*, and a sharp difference of no visible evidence of nematode attack versus visible evidence was expected on the basis of previous work (6); however, contamination of the culture of *M. incognita* by *M. incognita acrita* was suspected and confirmed[4] in the course of the experiments. *M. incognita acrita* usually incites small galls on roots of resistant peppers, which causes most individual plants to be placed in the "slight" class (6). Resistant and susceptible varieties were grown as checks in each test, and all individual plants were placed in one of the following disease classes: healthy, slight, moderate, or severe. Final interpretation of the data was made by using the check varieties in each test to determine the division of disease classes into resistant and susceptible groups.

Seeds were collected separately from all individual pods involved in a particular cross and the identity maintained throughout the generations produced. In the same manner, seed from individual F_1 or F_2 plants

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[2] Department of Plant Pathology and Physiology, Mississippi Agricultural Experiment Station.

[3] Grateful acknowledgement is made to A. L. Taylor, Head, Section of Nematology, Agricultural Research Service, U. S. Department of Agriculture, for supplying pure cultures of the nematodes used and directions for handling them.

[4] Identifications of the nematodes were made by J. N. Sasser, Plant Pathology, North Carolina Agriculture Experiment Station, Raleigh, N. C.

TABLE 1.—*Response of progenies of 5 crosses of root-knot-resistant (Santanka xS and 405B Mexico) and root-knot-susceptible peppers grown in nematode-infested soil*

Cross[a]	Generation[b]	No. resistant	No. segregating	No. susceptible	Expected basic ratio	P[c]
C30	F_1	102		0	Resistant	
(Santanka xS	F_2	35		5	3:1	0.05-0.10
× Ruby King)	F_3	5	11	9	1:2:1	0.30-0.50
	F_3—11 segregating lines	232		74	3:1	0.70-0.80
C33	F_1	40		48	1:1	0.30-0.50
(Santanka xS	F_2	74		27	3:1	0.50-0.70
× California	F_3	5	8	7	1:2:1	0.50-0.70
Wonder Special)	F_3—8 segregating lines	177		62	3:1	0.70-0.80
C35	F_1	51		48	1:1	0.70-0.80
(Santanka xS	F_2	66		28	3:1	0.20-0.30
× Truheart)	F_3	6	16	6	1:2:1	0.70-0.80
	F_3—16 segregating lines	245		113	3:1	<0.01
C37	F_1	102		94	1:1	0.50-0.70
(405B Mexico	F_2-plant 1	168		43	3:1	0.10-0.20
× Burlington)	-plant 2	30[d]		338	Susceptible	
	F_3-plant 1	2	4	0	1:2	
	-plant 2	1	6	0	1:2	0.20-0.30
	F_3—10 segregating lines	212		66	3:1	0.50-0.70
C38	F_1	63		73	1:1	0.30-0.50
(405B Mexico	F_2-plant 1	82		35	3:1	0.20-0.30
× Truheart)	-plant 2	87		33	3:1	0.50-0.70
	-plant 3	3		117	Susceptible	
	F_3	4	3	0	1:2	0.10-0.20
	F_3—3 segregating lines	58		23	3:1	0.30-0.50

[a] All F_2 and F_3 progenies in C30, C33, and C35 were derived from resistant F_1 plants. The F_2 progenies of C37 and C38 were from random untested F_1 plants and all susceptible F_2 plants from C37 and C38 were discarded before the F_3 generation was grown. The results show that heterozygous resistant parents were used in C33, C35, C37, and C38.

[b] All counts of F_1, F_2, and F_3 segregating lines are of individual resistant or susceptible plants and counts of F_3 are of resistant, segregating, or susceptible progenies.

[c] P value derived from Chi-square test for goodness of fit to the expected ratios.

[d] The number of resistant plants appeared too high to consider as escapes, and 7 were progeny tested and confirmed as resistant. Possibly a mechanical mixture of seed occurred.

was kept separate. Where results were the same, pod and plant identifications were deleted and results reported as the totals of a specific generation of a cross. There were no variations in results with seed from different pods in a cross, but in some cases it was necessary to retain identification of plant progenies in the F_2 and F_3.

RESULTS.—The numbers of resistant and susceptible individuals in F_1, F_2, and segregating F_3 progenies and the classification of F_3 progenies are presented in Table 1 along with the probable ratios. Examples of the results in F_1, F_2, and F_3 generations are shown in Figure 1. In crosses C30, C33, and C35, F_1 plants were inoculated and all susceptible ones were discarded. F_2 and F_3 generations were grown from resistant F_1 plants before further tests were made. In crosses C37 and C38, F_2 seed were collected from random F_1 plants before a nematode-resistance test was made. The F_2 generation was grown in nematode-infested soil and susceptible plants were discarded. Tests for nematode resistance of the F_1 and F_3 generations of C37 and C38 were made after F_3 seed was obtained. The results obtained from all crosses indicated that a single dominant gene determined inheritance of resistance to the root-knot nematodes involved.

All plants of the F_1 of cross C30 were resistant, and the F_1 of the other 4 crosses segregated in a 1:1 ratio. Evidently heterozygous resistant parents were used in these 4 crosses. Notes available on the crosses showed that C37 and C38 were made in midwinter and that no seed set was obtained on the commercial parents. All F_1 seed in both crosses were set on 1 vigorous plant labeled "405B Mexico" and described as having leaves that were larger and less pubescent than usual for the

→

FIG. 1. Peppers grown in soil infested with root-knot nematodes. Left (in each group), the resistant parent; right, the susceptible parent. The 2 middle plants in each were as follows: A) F_1 from C30 (Santanka xS × Ruby King), B) resistant and susceptible F_2 plants from C35 (Santanka xS × Truheart), C) homozygous susceptible F_3 from C35 (C35-5-4), and D) homozygous resistant F_3 from C35 (C35-5-6).

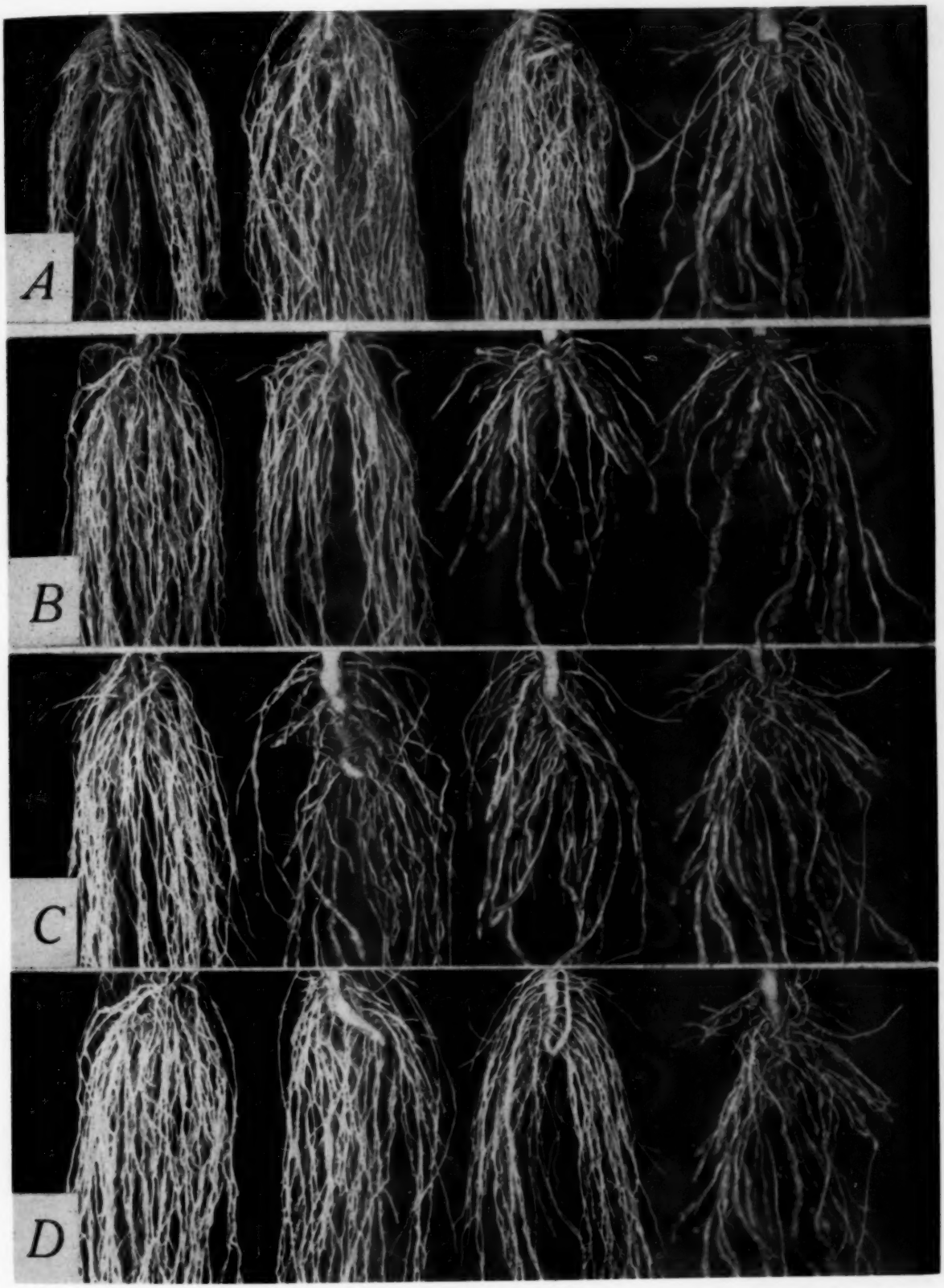
A
B
C
D

TABLE 2.—*Numbers of resistant and susceptible plants among progenies from crosses and backcrosses of selected root-knot-resistant and root-knot-susceptible pepper plants when grown in nematode-infested soil*

Cross	Generation	No. resistant	No. susceptible	Expected basic ratio	P[a]
Homozygous resistant F_2 plants from C37 and C38 (*NN*) × susceptible (*nn*)[b]	F_1	92	0	resistant	
Heterozygous resistant F_2 plants from C37 and C38 (*Nn*) × susceptible (*nn*)	F_1	47	59	1:1	0.20-0.30
Backcross 1 to susceptible (*Nn* × *nn*)[c]	F_1	21	28	1:1	0.30-0.50
Backcross 2 to susceptible (*Nn* × *nn*)	F_1	59	66	1:1	0.50-0.70

[a] P value derived from Chi-square test for goodness of fit to the expected ratios.
[b] Homozygous and heterozygous root-knot-resistant plants from C37 and C38 were determined by subsequent testing of F_3 progeny. C37 and C38 were previous crosses of resistant × susceptible plants. Susceptible plants were from the varieties Early California Wonder, Yolo Wonder, and Truheart.
[c] Backcrosses 1 and 2 were crosses of heterozygous resistant F_1 plants and susceptible plants from the commercial varieties listed in *b*.

strain. No records of difficulties in crossing were made for crosses C30, C33, and C35, which were made in late spring. Neither of the resistant parents was pure lined for resistance before the crosses were made.

Seven resistant F_2 plants of C37 and C38 were selfed and also crossed to commercial varieties at the same time. F_3 tests demonstrated that 3 of the plants were homozygous for resistance and 4 heterozygous. Crosses involving the 3 homozygous plants produced all resistant F_1 plants, and those involving the heterozygous plants produced F_1 plants that segregated 1:1 for resistance and susceptibility. The totals obtained are shown in Table 2 along with the results of 2 subsequent backcrosses to commercial varieties. This provided further confirmation of the single-dominant-gene hypothesis, since the resistance was recovered in the F_2 and maintained in 2 following backcrosses to recurrent susceptible parents. It is suggested that the symbol *N* be used to designate the dominant gene controlling resistance to *M. incognita* and *n* to designate the recessive allele controlling susceptibility.

After it was concluded that inheritance of the resistance to the nematodes used (primarily *M. incognita* with an undetermined amount of *M. incognita acrita*) is controlled by a single gene, tests of parents and resistant F_3 progenies were made. The resistant parents, the susceptible parents, and the resistant F_3 progenies from all combinations made except Santanka xS × Truheart were tested for reaction to *M. incognita*, *M. incognita acrita*, *M. arenaria*, and *M. javanica*. *M. hapla* was omitted since none of the varieties are resistant to that species. Stock seed was used of all va-

TABLE 3.—*Root-knot indices of parents and homozygous resistant F_3 progenies grown in nematode-infested soil*

Variety or strain	Index[a] when indicated species of *Meloidogyne* was used				
	M. incognita	*M. incognita acrita*	*M. arenaria*	*M. javanica*	None
Santanka xS	11.1[c]	9.6[d]	0	0	0
C30-4-6 (S × R)[b]	0	0.8	0	0	0
Ruby King	75.0	72.5	7.5	0	0
C33-3-3 (S × C)	5.8	8.3	0	0	0
C33-3-5 (S × C)	5.2	9.2	0	0	0
California Wonder Special	68.3	70.2	9.2	0	0
Early California Wonder[e]	75.0	72.4	57.5	23.3	0
405B Mexico-4H[f]	0	0	0	0	0
C37-0-6 (M × B)	0	0	0	0	0
Burlington	66.4	73.3	10.5	0	0
C38-0-3 (M × T)	2.5[g]	0	0	0	0
C38-0-7 (M × T)	0	0	0	0	0
Truheart	75.0	75.0	0	0	0

[a] The number of plants in each root-knot class was multiplied by the class value (healthy, 0; slight, 25; moderate, 50; severe, 75; and dead, 100), the totals added, and the sum divided by the total number of plants to obtain the root-knot index for each entry.
[b] Parentage of F_3 progenies given in parenthesis; S = Santanka xS, R = Ruby King, C = California Wonder Special, M = 405B Mexico, B = Burlington, and T = Truheart.
[c] One of 29 plants was susceptible, not included in index calculation.
[d] Four of 26 plants were susceptible, not included in index calculation.
[e] This variety was not involved in the crosses; used as susceptible control.
[f] This variety was pure lined for nematode resistance. It and all entries below it were tested in a separate experiment from all entries above it in the table.
[g] One off-type susceptible plant in a total of 30.

rieties from the same sources used in growing the parents for crossing, except that a strain pure lined for root-knot resistance (405B Mexico-4H) was substituted for 405B Mexico. Root-knot indices, based on 20 to 30 plants per entry, were prepared as described earlier (5) for each entry with each nematode (Table 3).

If it is assumed that the resistance expressed when the 6 homozygous F_3 progenies were selected was to *M. incognita* alone, in the species tests they were also resistant (indices 25.0 or less) in each case to *M. incognita acrita* and the indices were comparable to those of the resistant parents. Thus the same gene or 2 closely linked genes controlled resistance to *M. incognita* and to *M. incognita acrita*. The 6 F_3 progenies used in the species tests were picked at random from 26 that had been determined as homozygous resistant progenies in the nematode-resistance tests. Therefore, regardless of how much *M. incognita acrita* entered into the latter tests, the conclusions regarding the genes would be the same.

All 6 F_3 progenies were also resistant to *M. arenaria* and to *M. javanica*. All of the parents were resistant to *M. arenaria*, although there were differences in the degree of resistance. Root-knot indices of the F_3 progenies and of the resistant parents were zero, and indices of the susceptible parents were 7.5, 9.2, 10.5, and 0 (Table 3). The indices of both types of parents and of the F_3 progenies were zero with *M. javanica*.

Discussion.—The gene (or closely linked genes) identified here may have been concerned with resistance to root knot incited by *M. arenaria* and *M. javanica*, but no firm conclusions on that point can be drawn from the data. In 3 of the 5 crosses, the commercial parents did have low indices with *M. arenaria* whereas indices of resistant parents and of the F_3 progenies were zero. Comparison with the indices of the susceptible check variety (Early California Wonder) indicated that at least 1 other gene or set of genes was present where *M. arenaria* and *M. javanica* were concerned.

Behavior of the progenies from the crosses with the 2 different resistant parents was the same when grown under similar conditions. Apparently the gene or genes controlling resistance to *M. incognita* and *M. incognita acrita* were the same in both varieties; however, experiments to determine this point were not conducted.

Since random homozygous resistant F_3 progenies from crosses of resistant $\times$ susceptible were resistant to both *M. incognita* and *M. incognita acrita*, control of resistance by a single gene or closely linked genes was concluded. An alternative explanation could have been that the 2 nematodes were fundamentally the same, at least in regard to their parasitic action on pepper, and resistance was to 1 rather than to 2 separate entities. In previous work (6) there was a consistent difference in reaction of a resistant variety to the 2 nematodes.

In either case, it should be possible to breed for strains of commercial-type peppers resistant to 4 root-knot nematodes by using only 1 root-knot resistance test in each generation. This would require as parents a variety containing the *N* gene and a commercial variety selected for resistance to *M. arenaria* and *M. javanica*.

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CULTURAL VARIABILITY IN SEPTORIA AVENAE THROUGH SUCCESSIVE SINGLE-MACROSPORE TRANSFERS [1]

A. L. Hooker [2]

SUMMARY

Single-macrospore cultures of *Septoria avenae* on potato-dextrose agar in tubes varied in rate of growth, sporulation, and other cultural characteristics. Monospore subcultures from these also showed a high degree of cultural variability. The process of single-spore analysis through the establishment of 10–30 monospore subcultures from selected monospore cultures each vegetative generation was continued for 3–11 successive conidial transfers in 4 isolates of the pathogen, resulting in the observation of over 3800 monospore cultures. Within each of 4 initial monospore cultures of 1 isolate selected for detailed analysis, considerable variability was observed up to the third or fourth generation. Subsequent subcultures became more uniform, and types that remained stable were obtained. Some of these appeared suddenly as a single variant culture whereas others required several transfers for stabilization. A few cultures remained variable for at least 6 generations. Some cultures that were stabilized for morphological characteristics were stable after storage for 5 months at 10°C and after several mass-spore transfers had been made, whereas others were variable. Stable but new cultural types were obtained following successive single-spore transfers from these variable cultures. Passage through oat seedlings did not appear to increase or to decrease cultural variability. Two isolates of *S. avenae* were less variable than the others. In one of these, subcultures from spore germ-tube tips proved as variable as did subcultures from spores. Stabilized cultures derived initially from a single macrospore or from a germ-tube tip differed significantly in pathogenicity on oat seedlings in greenhouse tests. Isolates of *S. avenae* maintained in culture for 3 years were more variable upon single-spore analysis than were isolates maintained in culture for 1 year. It is concluded that monospore cultures of *S. avenae* may be variable but that a high degree of cultural stability can be obtained through successive single-spore transfers. The cultural variability observed during single-spore analyses in *S. avenae* could theoretically be due to a process of continuing mutation and subsequent vegetative association of different nuclear types within a heterocaryotic system and the attainment of cultural uniformity following several successive monoconidial transfers due to a chance dissociation of genetically alike nuclei during spore formation.

Septoria avenae Frank, the imperfect stage of *Leptosphaeria avenaria* G. F. Weber, infects the leaves, stems, glumes, and kernels of the oat plant. This Septoria disease has become established as a major oat disease in the northern and eastern regions of the United States and in eastern Canada.

Cultures of *S. avenae* from field material, established by transferring macrospores from pycnidia to potato-dextrose agar, have invariably been mycelial types with few if any pycnidia. On subculturing, however, sporulating strains can be found and selected. Single-spore culture technique was initially employed to determine if *S. avenae* expressed the so-called "dual phenomena" (1) and to establish uniform biotypes of the pathogen for use in pathogenicity studies. In this work, considerable variation in cultural characteristics was found. This variation was studied further, and the results are presented in this paper.

Few studies on variability in species of *Septoria* have been reported, although this phenomenon has been studied in many other pathogens. Johnson (4) observed differences in mycelial types in *S. avenae* and noted 2 sporulating variant types. The sporulating variants showed a marked tendency to revert to mycelial types upon subculturing. Results following a limited number of single-spore transfers (4) suggested that some sporulating variant cultures were culturally stable whereas others were not. Hansen (1) reported that many imperfect fungi, including some *Septoria* species, may exist in nature as 2 primary cultural types, i.e. mycelial and conidial. Intermediate types, which usually give rise to mycelial or conidial types upon single-spore analysis (1, 3), may be found. Hansen and Smith (3) and Hansen (1) have pointed out the advantages of single-spore analysis in studies of fungus variability and indicated that the "dual phenomena" as expressed by many fungi may be due to the presence of 2 genetically different nuclei, i.e. mycelial and conidial, in the thallus. The term heterocaryosis has been used to describe this (2, 3) or other conditions in which 2 or more genetically different nuclei exist in a cell, spore, or thallus.

MATERIALS AND METHODS.—Cultures of *S. avenae* used in these investigations were isolated from oat leaves. Isolates 400 and 427 were isolated in 1952 and Isolates 424 and 426 in 1954. All isolates were relatively nonsporulating initially, but sporulating cultures were obtained through constant selection for increased

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sporulation and through the use of spore transfers in subculturing. All were pathogenic on oat seedlings.

Single-spore transfers were made following the method of Hansen and Smith (3). A dilute macrospore suspension was prepared in sterile water and dispersed over the surface of 3 per cent agar containing a small amount of potato juice and dextrose in petri plates. Plates were tipped slightly so excess water drained from the agar surface and were incubated in this position overnight. The excess water was removed from the plates the following day, and single germinating spores were cut out readily with a sterilized needle under a dissecting microscope and transplanted individually to potato-dextrose-agar slants.

Subsequent single-spore transfers were made following 10–14 days of growth; unless indicated otherwise, these were made from monospore cultures. These successive single-macrospore transfers were made for several vegetative generations. All cultures established during the first cycle of single-spore transfers were regarded as T_1 (first transfer) cultures. T_1 cultures were indentified by letters (A, B, C, D, etc.). Cultures established following single-macrospore transfers from a T_1 culture were regarded as T_2 (second transfer) cultures. T_2 cultures were identified by the letter of the T_1 culture followed by a selection number (A-1, A-2, etc.). T_3, T_4, and succeeding generations of cultures were obtained and identified in a similar manner. All cultures in any 1 generation obtained following monospore transfers from the same "parent" monospore culture were regarded as a family. A T_2 family would therefore consist of all T_2 cultures obtained from the same T_1 culture. In a like manner, T_3, T_4, and subsequent families were obtained. Families were identified by the designation used to identify the monospore culture from which they were derived.

All tests were conducted on standard potato-dextrose agar and under ordinary laboratory conditions.

EXPERIMENTAL RESULTS.—*Isolate 400.*—Several macrospores were transferred from a single lesion in 1952 in establishing Isolate 400. It was maintained in the laboratory by mass-spore transfers for many generations prior to its study by single-spore analysis in 1955.

Thirty-eight single germinating macrospores from Isolate 400 were transferred to tubes of potato-dextrose agar. Resulting T_1 cultures were variable as to rate and shape of growth, color, pigmentation of substrate, sporulation, and other cultural characteristics (Fig. 1). Some cultures were nonsporulating. Eight sporulating T_1 cultures (A, B, C, D, E, F, I, and J) were selected for further single-spore analysis. The results obtained with 4 of these are summarized in Table 1. Although not studied as extensively, similar data were obtained with the 4 remaining macrospores.

Considerable variation in cultural type and sporulation was encountered among 173 monospore T_2 cultures obtained in the second successive transfer. Although the 8 original T_1 cultures differed in many morphological characteristics, these were not always

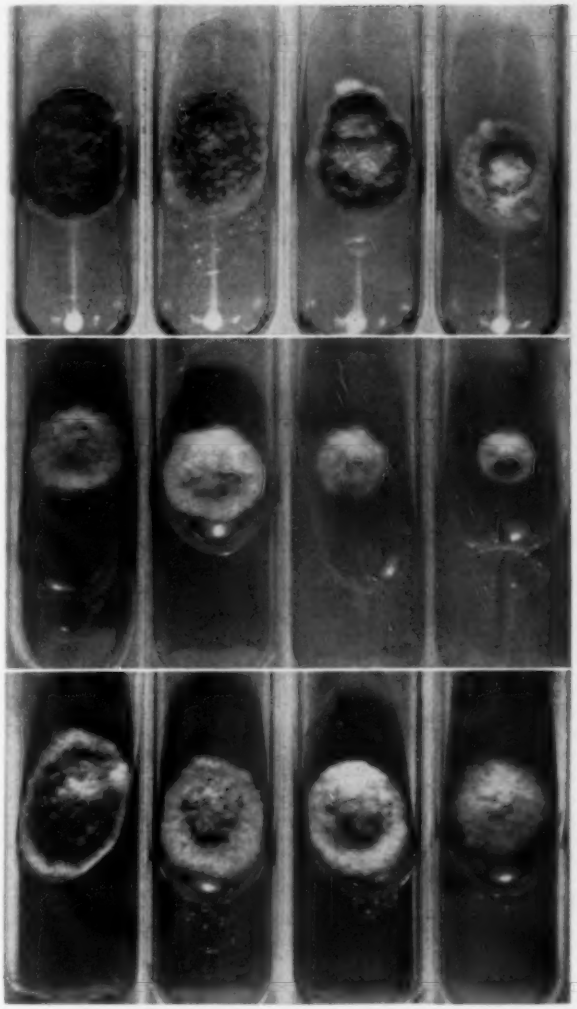

FIG. 1. Monospore subcultures from Isolate 400 of *Septoria avenae*, showing the range of cultural variability expressed following the first single-macrospore transfer. Cultures from left to right: top—A, B, C, and D; middle—E, F, G, and H; bottom—I, J, K, and L.

transmitted to their respective T_2 families. T_2 families obtained from strongly pycnidial T_1 cultures contained cultures with greater spore production than did those from poorly sporulating T_1 cultures. Only a few T_2 cultures were nonsporulating. Variation in cultural characteristics existed both within and between T_2 families. Variability decreased in subsequent transfers, and culturally uniform families were obtained. (Table 1).

Two rather distinct cultural types, one characterized by a bluish-green colony and substrate with many pycnida and the other by a reddish-brown colony and substrate with few pycnidia, occurred in the T_2 family of F. Both types were distinct from the mycelial type usually obtained upon first isolation. Four T_3 families (F-4, F-5, F-6, and F-7) derived from paired cultures

TABLE 1.—*Cultural variability observed during 8 successive single-macrospore transfers from 4 monospore cultures of Isolate 400 of Septoria avenae*

Parent culture[a]	Single-spore subcultures: Generation[b]	No.	Family[c] cultural characteristics and example illustrated in Fig. 2
A	T_3	25	Variable
A-1	T_3	15	Variable
A-2	T_3	13	Variable
A-3	T_3	15	Variable
A-1-1	T_4	14	Variable
A-1-2	T_4	2	Variable
A-2-1	T_4	10	Variable
A-3-1	T_4	10	Variable
A-1-1-1	T_5	9	Variable
A-1-2-1	T_5	10	Uniform
A-2-1-1	T_5	10	Uniform
A-1-1-1-1	T_6	12	Uniform
A-1-1-1-2	T_6	10	Variable
A-1-2-1-1	T_6	8	Uniform parental[d]
A-2-1-1-1	T_6	9	Uniform parental
A-1-1-1-1-1	T_7	10	Uniform parental (A-1-1-1-1-1-1)
A-1-1-1-2-1	T_7	4	Uniform parental (A-1-1-1-2-1-1)
A-1-2-1-1-1	T_7	3	Uniform parental (A-1-2-1-1-1-1)
A-2-1-1-1-1	T_7	9	Uniform parental (A-2-1-1-1-1-1)
A-1-1-1-2-1-1	T_8	9	Uniform parental
E	T_2	3	Variable
E-1	T_3	10	Variable
E-2	T_3	10	Uniform
E-1-1	T_4	10	Variable, 2 types
E-1-2	T_4	10	Uniform
E-2-1	T_4	4	Uniform parental
E-1-1-1	T_5	10	Uniform parental
E-1-1-2	T_5	13	Uniform parental
E-1-2-1	T_5	7	Uniform parental (E-1-2-1-1)
E-2-1-1	T_5	10	Variable, 4 uniform parental (E-2-1-1-1) and 6 with slightly restricted growth
E-1-1-1-1	T_6	10	Uniform parental
E-1-1-2-1	T_6	10	Uniform parental
E-2-1-1-1	T_6	10	Uniform parental
E-1-1-1-1-1	T_7	10	Uniform parental (E-1-1-1-1-1-1)
E-1-1-2-1-1	T_7	9	Uniform parental (E-1-1-2-1-1-1)
F	T_2	26	Variable
F-1	T_3	8	Variable
F-2	T_3	4	Variable
F-3	T_3	10	Uniform
F-4	T_3	31	Variable
F-5	T_3	33	Variable
F-6	T_3	30	Variable
F-7	T_3	30	Variable
F-1-1	T_4	15	Variable
F-2-1	T_4	15	Variable
F-3-1	T_4	2	Uniform
F-6-1	T_4	16	Variable
F-7-1	T_4	16	Variable
F-3-1-1	T_5	9	Uniform parental (F-3-1-1-1)
F-6-1-1	T_5	8	Variable, 2 types
F-7-1-1	T_5	10	Variable
F-6-1-1-1	T_6	9	Uniform parental (F-6-1-1-1-1)
F-7-1-1-1	T_6	10	Variable, 2 types (F-7-1-1-1-1 and F-7-1-1-1-2)
F-7-1-1-1-1	T_7	9	Uniform parental
F-7-1-1-1-2	T_7	10	Uniform parental
J	T_2	25	Variable
J-1	T_3	14	Variable
J-2	T_3	15	Variable
J-3	T_3	10	Variable
J-1-1	T_4	10	Variable
J-2-1	T_4	10	Variable
J-3-1	T_4	10	Variable
J-1-1-1	T_5	8	Variable
J-1-1-2	T_5	10	Uniform
J-1-1-3	T_5	9	Variable
J-1-1-4	T_5	10	Uniform
J-1-1-5	T_5	10	Variable, 2 types
J-3-1-1	T_5	10	Variable
J-3-1-2	T_5	10	Variable
J-3-1-3	T_5	10	Variable
J-1-1-1-1	T_6	10	Variable, 2 types
J-1-1-2-1	T_6	10	Variable
J-1-1-3-1	T_6	10	Variable
J-1-1-4-1	T_6	10	Uniform parental
J-1-1-5-1	T_6	9	Variable
J-1-1-5-2	T_6	10	Variable
J-3-1-1-1	T_6	10	Variable
J-3-1-2-1	T_6	10	Variable
J-3-1-3-1	T_6	10	Variable
J-1-1-1-1-1	T_7	8	Uniform parental (J-1-1-1-1-1-1)
J-1-1-1-1-2	T_7	7	Uniform parental (J-1-1-1-1-2-1)
J-1-1-2-1-1	T_7	6	Variable
J-1-1-3-1-1	T_7	3	Uniform
J-1-1-4-1-1	T_7	9	Uniform parental (J-1-1-4-1-1-1)
J-1-1-5-1-1	T_7	9	Uniform parental
J-1-1-5-2-1	T_7	10	Uniform parental (J-1-1-5-2-1-1)

[a] All were monospore cultures from which single-spore subcultures were established. Monospore cultures established from Isolate 400 were indicated by the letters A, E, F, and J. Cultures A-1, A-2, and A-3 were monospore subcultures derived from culture A. Cultures A-1-1 and A-1-2 were monospore subcultures derived from culture A-1. Other cultures were derived and identified in a similar manner.

[b] Indicates the transfer cycle of the monospore subcultures; i.e. T_1 cultures were established from Isolate 400 during the first cycle of single-spore transfers; T_2 cultures were established from T_1 cultures; T_3 from T_2; etc.

[c] All cultures in any 1 generation obtained following monospore transfers from the same "parent" monospore culture.

[d] All cultures were alike and morphologically similar to the monospore culture from which they were derived.

of each type resembled the parental type in these characteristics but were variable in other aspects.

A variable family derived in the T_4 generation from culture E-1-1 (Table 1) was composed of 9 uniform and 1 variant culture. Subcultures derived from cultures E-1-1-1 and E-1-1-2, representing each type, were

uniform and morphologically similar to their respective parents. A similar case occurred in the family F-7-1-1-1. Seven T_6 cultures were largely pycnidial with a dark mycelial overgrowth, and 3 cultures were largely pycnidial with a light mycelial overgrowth. The dark mycelial overgrowth had been the typical type in the 2 previous generations. Subcultures in the T_7 generation were uniform for each cultural type (Table 1).

Paired cultures illustrating the morphological characteristics of 16 families that were uniform as to cultural type are illustrated in Figure 2. Each illustrates the characteristic morphological type expressed when a family became uniform in any generation. All appeared to be distinct and stable for their respective morphological characteristics. As indicated by their selection numbers, each set of 4 paired cultures were subcultures from 4 different T_1 cultures and in turn from 4 different macrospores. Each illustrates a portion of the cultural variability obtained following successive single-macrospore transfers from a monospore culture.

In most cases, the amount of variability within families became less and the differences between families became greater with each successive generation of monospore transfers. Many families became uniform and once this was attained continued to be so upon further spore transfers. A few families were uniform except for 1 or a few variant cultures, and within a family these variants usually were alike. These variant cultures frequently showed a color difference or less growth than the others, but all continued to sporulate abundantly. Subcultures, established by transferring single macrospores from variant cultures within an otherwise uniform family usually were uniform and resembled their respective variant parent. Subcultures from the predominant parental types were uniform, and in no case did the same variant types reappear. Subcultures from uniform families continued to be uniform and resembled their respective parental types with few exceptions. Other families, however, continued to be variable after 6 or 7 generations of successive monospore transfers. Many variable families resembled their parental types in certain morphological characteristics and were uniform in this respect but variable in others.

Isolate 427.—Isolate 427 was maintained in culture for 3 years prior to its study by single-spore analysis. It was primarily a mycelial culture but produced a few pycnidia with spores. Twenty-seven T_1 cultures proved to be variable in cultural characteristics but less variable than T_1 cultures from Isolate 400. All 15 T_2 families, comprising 106 T_2 cultures, were variable, but many sporulated better than did the original Isolate 427. T_3 families comprising 128 T_3 cultures were established from 16 T_2 cultures. Many of these T_3 families assumed quite distinct cultural characteristics and produced numerous pycnidia, but only 3 were relatively uniform. Cultural characteristics of T_2 families frequently were expressed in subsequent T_3 families.

The analysis of Isolate 427 was discontinued after the third successive transfer, as the data obtained were similar to those for Isolate 400.

Isolate 424.—Isolate 424 was isolated from an oat leaf in December, 1954. Two macrospores from a single pycnidium were transferred in establishing the culture. It was composed primarily of mycelium through successive transfers until March, 1955. Sporulating cultures were highly pathogenic on oat seedlings and adult plants. In November, 1955, a study was initiated to investigate pathogenic variability of Isolate 424 through successive monospore subcultures from a single macrospore. In addition, notes on cultural variability as summarized in the following sections and in Table 2 were taken. Selections for transfers within families, however, were based upon pathogenicity tests and not on cultural characteristics.

Thirty-five of the 39 T_1 cultures established from Isolate 424 appeared to be morphologically uniform. The remaining 4 cultures differed slightly in the amount of growth and in the proportion of mycelium to pycnidia. From 1 to 2 variant cultures were found in each of 7 T_2, 5 T_3, 8 T_4, 1 T_5, 4 T_6, and 4 T_7 families. Four T_3, 5 T_4, 3 T_5, and 1 T_6 family were still more variable, with as many as 4 cultural types in each. T_4 subcultures from a variable family were more variable than were subcultures from a uniform family. Two uniform T_5 families were from variant T_4 cultures and were morphologically similar to these variant types. In a similar manner, 2 T_7 families derived from variant cultures were of a distinct morphological type. The lowest percentage of variant type cultures was found in the T_7 generation, and the largest percentage of uniform families was found in the T_5, T_6, and T_7 generations.

Isolate 424 was considerably less variable in cul-

TABLE 2.—*Cultural variability observed in successive single-macrospore transfers from Isolate 424 of Septoria avenae* [a]

Generation [b]	No. of families [c]		No. of single-spore subcultures	
	Uniform	Variable	Parental [d]	Variants
T_1	0	1	35	4
T_2	8	7	114	13
T_3	8	9	140	31
T_4	7	13	164	29
T_5	15	4	113	8
T_6	15	5	114	12
T_7	16	4	129	5

[a] T_1 cultures were established from Isolate 424; T_2 families from each of 15 T_1 cultures; T_3 families from each of 17 T_2 cultures, all of which were derived from the same T_1 culture; T_4 families from each of 20 T_3 cultures, all of which were derived from 2 T_2 cultures; T_5 and succeeding families, each from monospore cultures in the preceding generation all of which were derived from 4 cultures in the next preceding generation.

[b] See second footnote, Table 1.

[c] See third footnote, Table 1.

[d] Cultures were classified as parental if they were morphologically similar to the monospore culture from which they were derived. Parent monospore cultures were frequently of diverse types.

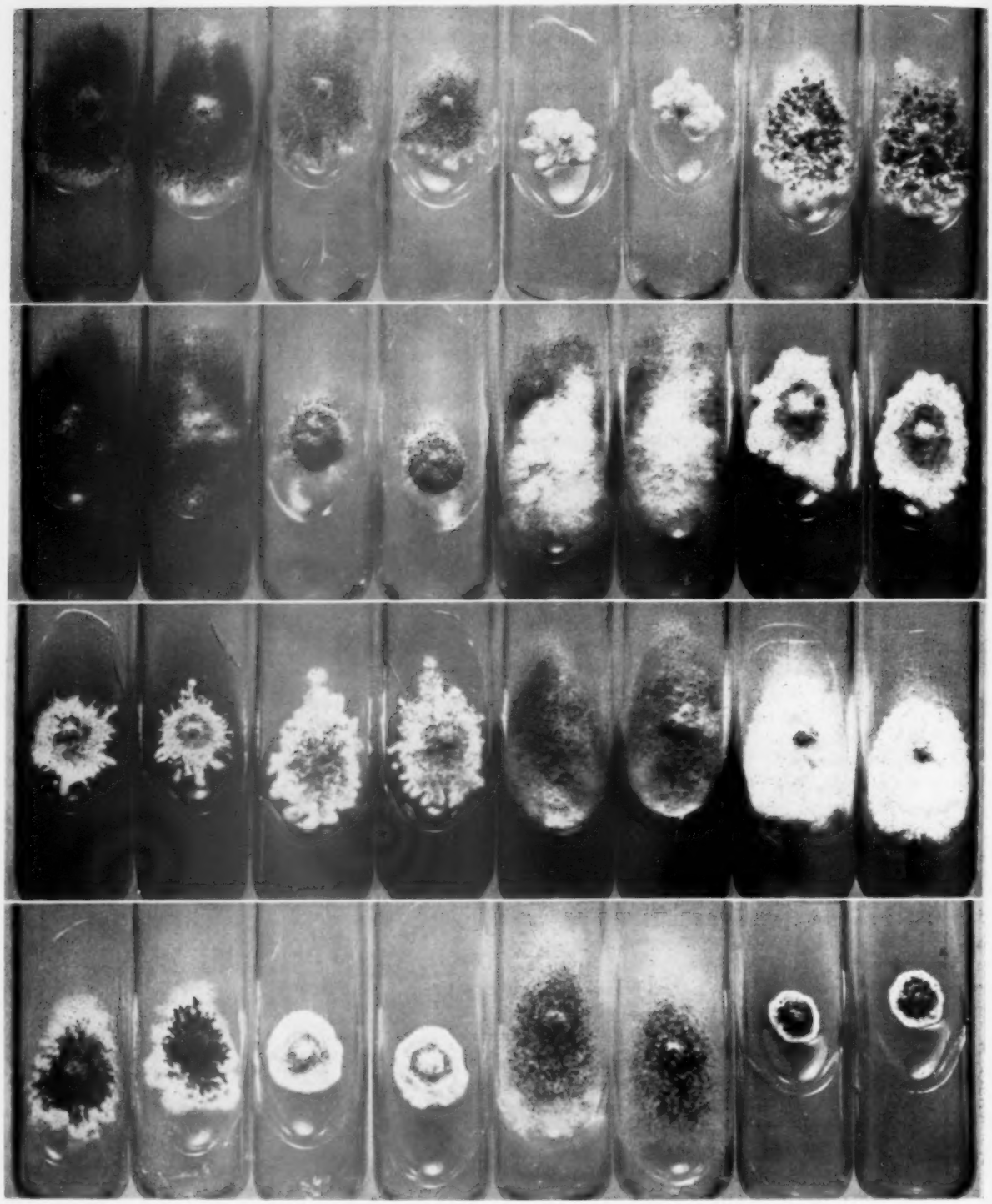

FIG. 2. Representative monospore cultures of uniform families obtained following 5–7 successive single-macrospore transfers from 4 monospore cultures in Isolate 400 of *Septoria avenae*, illustrating a range of cultural types; a portion of the variability expressed among subcultures derived from the monospore cultures A, E, F, and J (Fig. 1); and the degree of unformity following the successive transfers. Paired cultures from left to right: First row—A-1-1-1-1-1-1, A-1-2-1-1-1-1, A-1-1-1-2-1-1, and A-2-1-1-1-1-1; second row—E-1-1-1-1-1-1, E-1-1-2-1-1-1, E-1-2-1-1, and E-2-1-1-1; third row—F-3-1-1-1, F-6-1-1-1-1, F-7-1-1-1-1, and F-7-1-1-1-2; fourth row—J-1-1-1-1-1-1, J-1-1-1-1-2-1, J-1-1-4-1-1-1, and J-1-1-5-2-1-1. The monospore cultures established from Isolate 400 are designed by the letters A, E, F, and J; numbers following each letter indicate the monospore culture selected for subculturing in each vegetative generation of lineal descent.

tural characteristics than were Isolates 400 and 427. Cultures for continued transfers in Isolate 424, however, were chosen on the basis of pathogenicity, and many variant types were discarded without further study. Variant cultures, if selected for further study, usually produced uniform subcultures of the variant types. A majority of the cultures established in the study were of a uniform morphological type, and most of the variation expressed was in the form of a few variant cultures in an otherwise uniform family.

Isolate 426.—Isolate 426 was also isolated from an oat leaf in December, 1954, and was established by transferring a single macrospore from a pycnidium. This isolate was highly pathogenic on oats and sporulated well in successive transfers after March, 1955. In December, 1955, a study of the variability encountered in subsequent single-spore transfers from cultures established by transferring hyphal tips from opposite ends of germinating macrospores and from the remnant spore body was initiated.

Hyphal tips from germ tubes of opposite end-cells of 5 germinating macrospores and the germinating spore remnant in each case were transferred to tubes. All T_1 cultures appeared morphologically alike, and 6 resulting from the 3 transfers from 2 spores were selected for further analysis.

The proportion of variant to parental type cultures in the second to the fifth transfer cycle from the 6 selected cultures is indicated in Table 3. The parent monospore cultures for all families and generations summarized in Table 3 were morphologically alike. When transfers from all of these cultures in all generations and families were considered, 5.10 per cent of the subcultures originating from hyphal tips and 2.27 per cent of those originating from remnant spores were of various variant types. This difference, however, was not statistically significant.

Single-spore subcultures were occasionally established from variant types. A T_3 family from a variant T_2 culture characterized by more mycelium than characterized the parent was composed of 16 cultures similar to the predominant cultural type of Isolate 426 and of 4 cultures similar to the parent T_2 variant. All of the T_3 cultures from a second T_2 variant culture were of the cultural type typified by Isolate 426. One T_4 family from a variant T_3 culture characterized by a semirestricted growth and abundant pycnidial production was slightly variable, but all cultures were similar to the variant parent in cultural characteristics. An additional T_4 family, established from a variant T_3 culture characterized by a high proportion of mycelium to pycnidia, was comprised of 7 cultures similar in type to the variant parent and 4 that were nonfruiting. One T_4 variant culture, characterized by tenuous growth, originated from a remnant-spore transfer in T_1. A T_5 family established from this culture was uniform for the variant type. Five T_4 cultures originating from hyphal tip transfers in T_1 were of various variant types characterized by differences in degree of growth, in proportion of mycelium to pycnidia in the culture, in color and amount of pigment produced in the substrate, and in other characteristics. T_5 families from these variant T_4 cultures resembled their respective parents in cultural characteristics. One of these T_5 families was uniform, 1 was uniform except for 1 variant culture (additional type), and 3 were variable and consisted of several types.

Spore analysis following storage.—Monospore cultures of respresentative families of Isolate 400 were stored at 10°C from May to September. Following several mass-spore transfers, 15 cultures were subjected to single-spore analysis. Two of the families represented had been variable and 13 had been uniform prior to storage. One hundred forty single-spore subcultures were established. In addition, 24 monospore cultures were established from a mass culture of the original isolation (Isolate 400).

Subcultures from only 4 of the families were uniform and resembled their respective parental types. Subcultures from 1 family were uniform but differed slightly from the parental type. The subcultures were variable in 4 families, but in each case included representatives of the parental type. Subcultures from 3 families were of only 2 or 3 types in each case, but none of those resembled the parental type. In the remaining families, 4 or more cultural types were expressed among the subcultures. The monospore subcultures from Isolate 400 showed a wide degree of variation. The families that were variable prior to storage were among those showing the greatest degree of variability following storage.

Mass-spore transfers were made from monospore cultures of each parental type and from a selected number of the new cultural types. Three hundred forty-two single-spore cultures were established from 36 of these mass transfers. Fourteen such families, including 3 that were uniform before and immediately after storage, were uniform. Seven families were uniform except for 1 or 2 variant cultures; 15 families were variable, being composed of 3 or more cultural types.

Another generation of single-spore transfers was

TABLE 3.—*Proportion of variant to parental type cultures in the second (T_2) to the fifth (T_5) transfer cycles from cultures established by transferring hyphal tips and spore-remnant of 2 germinating macrospores of Isolate 426 of Septoria avenae*

Origin of T_1 culture	Proportion of variant to parental[a] type cultures			
	T_2	T_3	T_4	T_5
Hyphal tip 1 of spore 1	0/15	1/19	0/20	1/14
Hyphal tip 2 of spore 1	1/15	0/20	0/20	5/15
Remnant of spore 1	1/15	0/21	1/19	0/15
Hyphal tip 1 of spore 2	0/15	0/20	0/20	0/12
Hyphal tip 2 of spore 2	2/11	1/20	2/19	0/13
Remnant of spore 2	0/10	1/20	0/20	0/15

[a] Parent monospore cultures in all generations and families were morphologically alike.

made in 10 families. All were originally derived from the same T_1 culture (E). One hundred twenty-five monospore cultures were established. Two families continued to be uniform, 3 continued to be variable, and 5 that previously had been variable were uniform. In 2 of last 5 cases, the previous generation was uniform except for 1 or 2 variant cultures.

Ten of these families were subjected to another generation of single-spore analysis. Single macrospore transfers, therefore, except for storage and mass transfers, had been made for 11 successive generations in 8 families, for 10 generations in 1 family, and for 9 generations in the remaining family at the end of this series. Of these, 8 families were uniform and 2 proved to be variable as to cultural type.

Ten morphologically distinct cultures, each of which produced uniform families of 10–15 monospore subcultures, were obtained in the single-spore analysis of the monospore T_1 culture E from Isolate 400. Other morphologically distinct and uniform cultures were obtained in the single-spore analysis of the T_1 cultures A, F, and J. These all behaved as genetically uniform clones. Some additional families, however, continued to be variable even though single macrospores had been transferred for 7 or more successive vegetative generations.

Spore analysis following host passage.—Susceptible oat seedlings were inoculated with cultures from 7 T_5, T_6, or T_7 families of Isolate 400. These families had been in storage at 10°C from May to September and mass transferred several times. The reisolations from the host were subjected to single-spore analysis in a parallel series with cultures maintained in tubes. Two hundred twenty-seven cultures were observed. In each family, the cultural type and degree of variability of subcultures from the reisolations closely resembled those characteristics of subcultures from the companion cultures maintained in tubes. These observations were confirmed by another generation of single-spore transfers in 3 families from parallel cultures before and after host passage. Passage through the host did not appear to increase or to decrease the amount of cultural variability.

Pathogenicity tests.—Numerous pathogenicity determinations of different isolates of *S. avenae* were made in replicated experiments in the greenhouse on 5 oat varieties (Branch, Fayette, Minland, Rodney, and Sauk) known to have different levels of resistance to *S. avenae* under natural conditions in the field. Seven- to 12-day-old sporulating cultures on potato-dextrose agar were blended with water in a Waring Blendor for 1 minute at the rate of 1 petri plate of culture and substrate per 100 ml water. The inoculum was passed through 1 thickness of cheesecloth and sprayed on the plants with an atomizer at 15 lb. pressure. Inoculated plants were then placed in a moist chamber with abundant moisture for 36 hours. Lesions appeared in 3 days, and percentages of leaf area with symptoms were recorded after 7–9 days. The data obtained were subjected to statistical analyses.

Selected cultures obtained after 4 generations and after 6–8 generations (both before and after storage for 6 months at low temperature) of single-spore transfers from Isolate 400 were tested for virulence in the greenhouse on the 5 oat varieties in comparison with the original Isolate 400. In each of the 3 tests, statistically significant differences in virulence were measured among the cultures tested. Relatively low levels of infection were obtained with many cultures, but several subcultures obtained following successive single-spore transfers were more virulent than Isolate 400. It would appear that a separation of factors for virulence as well as for cultural characteristics had occurred during the spore transfers.

Pathogenicity comparisons between cultures reisolated from the host and those maintained in tubes were made on the 5 oat varieties. Seven paired cultures of Isolate 400 obtained following 5–7 generations of successive single-spore transfers were among those used. Significant differences in virulence were found among the 7 cultures, but the cultures after host passage as a group did not differ significantly in virulence from those maintained in tubes. Similar results were obtained with other isolates. These data indicate that host passage alone does not change significantly the virulence of cultures of *S. avenae*.

Also tested for pathogenicity were 15 cultures of Isolate 426 obtained following 4 generations of successive single-spore transfers from T_1 cultures established by transferring hyphal tips from germinating spores or by transferring the remnant spores. Statistically significant differences in pathogenicity were measured among the 15 cultures, but these were not associated with the source of the original transfers. Cultures derived from different remnant spores, from germ tubes of opposite end-cells of the spores, or from the same hyphal tip differed significantly in pathogenicity. Significant differences in pathogenicity were measured among cultures resembling Isolate 426 in cultural characteristics and among the various variant types.

Discussion.—*Septoria avenae* is a variable fungus that produces numerous cultural types on artificial media. Cultures isolated from naturally infected host material are composed primarily of mycelium despite the fact that secondary spread of the pathogen occurs primarily by spores. The proportion of pycnidia to mycelium usually increases if mass-spore transfers are made in subculturing until the culture is composed largely of pycnidia. This change from mycelial to pycnidial cultural type is largely independent of substrate and other environmental conditions and is assumed to be genetically determined. The existence of predominantly mycelial strains and predominantly pycnidial strains is suggestive of the "dual phenomenon" as reported by Hansen (1). In *S. avenae*, however, successive single-spore transfers have also revealed many different cultural types most of which have produced numerous pycnidia and spores.

A process of continuing mutation within a heterocaryotic system may be considered as the most feasible explanation for the variability encountered in the successive single-spore transfers of *S. avenae*. There is the rare possibility that somatic recombinations arising from the chance formation of heterozygous diploid nuclei could have occurred (5, 6). Other possibilities are mutation alone or some type of cytoplasmic inheritance. Because of the low frequency with which somatic recombinations have been found in other fungi (5, 6), it is unlikely that this can be regarded as accounting for a major portion of the variability in *S. avenae*. Cytoplasmic inheritance may account for a portion of the variability, but some form of nonrandom distribution of cytoplasmic constitutents during spore formation and subsequent development would be necessary to account for the data obtained. Some mechanism for a reduction in mutation rate each generation of single-spore transfer would be needed to account for the greater variability within T_1 and T_2 families than within T_6 and T_7 families if mutation alone is to be regarded as the sole explanation of the variability encountered. These arguments tend to rule out all hypotheses except that of mutation within a heterocaryotic system.

The gradual reduction in cultural variability and the attainment of stable types with successive single-spore transfers would be expected in a heterocaryotic fungus. Each cell of the macrospore of *S. avenae* contains a single nucleus (7), which presumably is haploid; if more than 1 nucleus enters a spore during its formation, a monospore culture could be comprised of as many as 4 genetically different nuclei. Chance assortment of nuclei could be expected to occur during spore formation in a monospore culture, and some spores could be formed with less than the maximum number of genetically different nuclei. This vegetative separation of genetically different nuclei could be expected to continue during successive single-spore transfers until spores with only 1 nuclear type are formed. Such cultures should then be stable and uniform upon subculturing. The foregoing is true of course if it is assumed that genetic factors in the nucleus determine cultural type and that no mutation or other intranuclear changes take place.

Mutation may account for a portion of the cultural variability in *S. avenae*. This is indicated by the appearance of single variant cultures that were immediately stable during the successive spore transfers and by the appearance of new types in stable cultures following storage. If these variant types were purely the result of vegetative nuclear assortment in a heterocaryotic culture, such a heterocaryotic culture on subsequent transfers would be expected to separate into its component parts or at least to continue to form the same variant culture from time to time. In no instance did this occur, and in no instance did the variant culture revert back to the "parental" type. The recovery of different distinct cultural types upon subsequent successive single-spore transfers from germ-tube-tip isolations could also be due to mutation. Presumably, all of the nuclei in the germ tube from any 1 cell of the macrospore arise from mitotic divisions of the single nucleus in that cell and, without mutation, would be genetically identical.

The greater variability encountered in Isolates 400 and 427, which had been maintained in culture for 3 years, than in Isolates 424 and 426, which had been maintained in culture for only 1 year, could be due to the greater accumulation of mutations in the former during the longer period in culture. Many of these mutations could have a selective advantage in culture, or at least no selective disadvantage; such nuclei could increase in culture whereas comparable mutations in nature might not persist. Mutations such as these could have occurred in the stable T_6 and T_7 families of Isolate 400 in storage and during the mass transfers following storage. The dense growth during the mass-spore transfers would have provided many opportunities for hyphal anastomoses and mixing of nuclei to occur. The reduction of cultural variability and the attainment of several uniform cultural types during successive single-spore transfers from these new variable cultures indicated that the chance assortment of genetically identical nuclei into individual macrospores occurred again.

The fact that subcultures derived following successive single-spores transfers differed significantly in pathogenicity indicates that in addition to (or along with) a separation of factors for cultural characteristics, a separation of factors for pathogenicity also occurred. Some of these factors may affect both cultural and pathogenic characteristics of the fungus whereas others may be independent. The mechanism permitting changes affecting pathogenicity during vegetative growth of the fungus may have some biological significance enabling the fungus to cope with a greater diversity of host genotypes.

The data indicate that a single macrospore transfer does not necessarily result in the establishment of a genetically uniform culture of *S. avenae*. This is particularly true of isolates which have been maintained in culture for several years. Successive single spore transfers, however, have resulted in uniform cultural types except for occasional variants. The uniform types presumably are due to the chance separation of genetically alike nuclei in multinucleate spores from a heterocaryotic mycelium, although they could be balanced heterocaryons or derive their stability through some other genetic means.

Cytological evidence of the entrance of genetically different nuclei into a macrospore during its formation and of hyphal anastomoses with the exchange and subsequent mixing of genetically different nuclei is lacking. Until these observations are made or until marker genes are available, *S. avenae* cannot for certain be regarded as a heterocaryotic fungus in the usual sense, although the cultural evidence is strongly suggestive.

The appearance of the variants and the numerous cultural types, however, are manifestations of the genetic variability of *S. avenae*.

The technique of successive monoconidial transfers for several vegetative generations may have value in establishing genetically uniform biotypes within heterocaryotic fungi.

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CULTURAL STUDIES WITH DORYLAIMUS ETTERSBERGENSIS [1]

John P. Hollis

SUMMARY

Dorylaimus ettersbergensis de Man 1885 was cultured, in Petri plates containing water agar, on a variety of microorganisms, including a blue-green alga (*Chroococcus* sp.), green algae (*Chlorella vulgaris* Beijer. and *Tetraedron* sp.), a protozoan (*Drepanomonas* sp.), and a fungus (*Cephalothecium* sp.). The nematode inserted its spear and fed on vegetative cells of the algae, on cysts of the protozoan, and on conidia of the fungus. Feeding involved the forcible removal of protoplasm from the cell, and its duration in an individual cell was determined by the type of microorganism attacked. *D. ettersbergensis* fed also on other nematodes. Picked specimens of *D. ettersbergensis*, disinfested of surface microorganisms except bacteria, were cultured on pure populations of the individual host microorganisms. Algae were the most congenial hosts, and the blue-green alga *Chroococcus* sp. was superior to the green algae in promoting and maintaining high populations of the nematode.

Dorylaimus Dujardin 1845, the largest genus of free-living nematodes, comprises some 200 species widely distributed throughout the world in water and soil (16). The genus is of particular ecological interest because of the diverseness and distribution of its species. In soils, *Dorylaimus* spp. usually occur in complexes of 10–20 species, which in aggregate form a major component of the microfauna. Overgaard Nielsen (14) summarized and extended the knowledge of these and other free-living nematodes, but his comprehensive monograph serves primarily to point out the gaps in our knowledge of their relations with the complex of microorganisms and higher plants in the soil.

The genus is related taxonomically to genera of proved phytopathological importance and wide geographical distribution. Speculations on the possible etiological role of *Dorylaimus* spp. in plant disease can stem from 1) deductions involving the comparative morphology of known pathogens, 2) occurrence in soil about the roots of plants, and 3) occurrence in plant tissues (15). There is no experimental evidence, however, of any species of *Dorylaimus* being implicated in the production of a plant disease, and their pathological significance remains an open question.

Linford and Oliveira (9), in summarizing the information on feeding habits of *Dorylaimus* species, stated that they had observed 10 species of *Dorylaimus* and several closely related nematodes of the genera *Discolaimus* and *Actinolaimus* feeding on other nematodes. Based on evidence previously reported and on their own observations, they stated that *Dorylaimus* species feed also on nematode and mite eggs and on oligochaet worms. In a later paper, Linford (8) added rotifers and infusoria to the host list.

Dorylaimus ettersbergensis de Man 1885, a common member of the genus, has been isolated and maintained in Louisiana State University laboratories in plates of water agar on various host microorganisms, including 3 algae, a protozoan, and a fungus (6, 7). Recently, the nematode has been observed feeding on other nematodes.

A detailed outline of the methods of isolation, disinfestation, and culturing of *D. ettersbergensis* on its

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microbial hosts is presented in this paper. Attention is given to the manner of separation and culturing of the individual host microorganisms and of the feeding activities of the nematode on the different hosts. The relative effectiveness of pure populations of the individual host microorganisms in promoting and maintaining high nematode populations was determined by comparative cultural experiments.

METHODS.—*D. ettersbergensis* was extracted for the first time in July, 1955, from soil collected in an experimental plot of oats at Homer, Louisiana. For the most part, the nematode has been cultured on a mixture of the host microorganisms in 3 per cent water agar, but at present it is being maintained largely in cultures of *Chroococcus* sp. in both water agar and a special algal agar (5).

Extractions and counts.—The absence of males renders *D. ettersbergensis* a useful choice for laboratory study because it simplifies procedures of transfer and the maintainance of cultures. The nematodes were extracted from 3 per cent water-agar cultures by the following method. The agar was diced with a scalpel into blocks 1–5 mm square; water was added and the suspension thoroughly mixed, then decanted into the muslin filter of an extraction funnel. The nematodes worked their way out of the agar blocks, passed through the muslin, and collected in the neck of the funnel, where they were drawn off 24 hours after preparation of the extraction mixture.

A Syracuse watch glass, marked with cross rectangles, was used for counting the nematodes extracted from individual Petri plate cultures. The rectangles comprised 1/7 of the total area of the watch glass, and only 2 passes across the watch glass were necessary for scanning the marked area under the dissecting microscope. These narrow rectangles permit more accurate counting than do circles of equal area in the central part of the watch glass; in the rectangles, the radial variations in nematode distribution are included and averaged in each count.

Preparation of new cultures.—The host microorganisms passed through the muslin filter with the nematodes and were transferred with them in water suspension to plates containing sterile agar. In initial phases of the work, the populations of nematodes in new cultures have been quite variable. This was due primarily to a lack of control over the microbial composition of the cultures. Culture of the nematode in large numbers during this period was assured by the direct transfer of large pieces of agar containing both nematodes and host microorganisms to new agar plates.

Partial disinfestation of D. ettersbergensis.—Thirty fungicidal and bactericidal materials were tested for efficiency in freeing the nematode of surface-contaminating and host microorganisms. Large numbers of nematodes were incubated for 24 hours in 1 per cent solutions or in the filtrates from 1 per cent suspensions of the materials and then examined for movement. Filtrates of several thiocarbamate fungicides of low solubility in water, of technical captan (92% *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide),[2] of Captan 50-W (50% captan), and of Spergon (48% tetrachloro-*p*-benzoquinone) eliminated most fungus contaminants and were nontoxic to the nematode.

Streptomycin sulfate and penicillin G (sodium) were tested for effectiveness in reducing bacterial contamination of the nematodes in culture. Streptomycin, at a concentration of 0.1 per cent, was toxic to the nematode when the incubation period was 24 hours but was not when it was 2–4 hours. Incubation of the nematodes for 2–4 hours in a 1 per cent solution of penicillin, following a 2-hour treatment in streptomycin solution or in mixtures of the 2 antibiotics, produced no harmful effects.

The following disinfestation procedure was adopted for all cultural experiments. The nematodes were washed by centrifugation and resuspension and then incubated in a thin layer of the appropriate solution in 50-mm Petri plates. Incubation was for 24 hours in filtrates of 1 per cent technical captan or Captan 50-W and then for 2 hours in 0.1 per cent streptomycin sulfate. These procedures were followed by 3 serial pickings of individual specimens to sterile water for the express purpose of eliminating algal cells and protozoan cysts that are resistant to the chemicals employed.

Additional treatment of the nematodes in solutions of bacteriocidal antibiotics failed to eliminate all bacteria. Transfer of treated nematodes to nutrient broth revealed consistently the presence of a residual bacterial flora.

Separation and culture of host microorganisms.—*Cephalothecium* sp. was maintained in pure culture and transferred in conidial suspension to agar plates for nematode culture. Algae were found occurring naturally in certain of the mixed cultures maintained for propagation of the nematode. These included the blue-green alga (*Chroococcus* sp.) and the green algae (*Chlorella vulgaris* Beijer. and *Tetraedron* sp.). Washings from these cultures contained also *Cephalothecium* sp., *Drepanomonas* sp., and bacteria; the disinfestation procedures were applied to eliminate the fungus and most of the bacteria. The algal cells and protozoan cysts were unaffected by these treatments. Cultures of the 2 species of green algae were found free of other host microorganisms and were cultured with associated bacteria on 3 per cent water agar. *Chroococcus* sp. and *Drepanomonas* sp. were found always mixed with the green algae. Treatment of the washings from these mixed cultures at a temperature of 50° C for 10 minutes eliminated the green algae, leaving a binary mixture of *Chroococcus* sp. and *Drepanomonas* sp. Pure populations of these organisms, in association with bacteria, were initiated by picking individual cells and transferring them to water agar in Petri plates.

[2]The sample of technical captan was furnished by Dr. James B. Bowers of the Stauffer Chemical Co., Agricultural Research Laboratory, Mountain View, Calif.

RESULTS.—*Feeding activities of the nematode.*—*D. ettersbergensis* has been observed repeatedly to feed on several different microorganisms (6, 7). These include the globose vegetative cells of a blue-green alga (*Chroococcus* sp.), 2 species of green algae (*Chlorella vulgaris* and *Tetraedron* sp.), the globose cysts of a ciliated infusorian (*Drepanomonas* sp.), and the ovoid conidia of a fungus (*Cephalothecium* sp.). Recently, in several instances, *D. ettersbergensis* has been observed feeding on other nematodes.

The mechanism of feeding appeared to be similar on all host microorganisms, involving a mechanical removal of protoplasm from the cell. There was no indication of a secretion of esophageal fluids or digestion of cellular contents prior to their ingestion by the nematode. The nematode placed its head in contact with the cell surface and pushed against it, frequently moving the cell; meanwhile, its spear or odontostyle was projected by rapid jabs until entrance had been effected. This jabbing was accompanied frequently by a rapid movement of the head from side to side, which produced a rasping action with the spear and appeared to facilitate its entrance into the cell.

The spear was inserted into algal cells and protozoan cysts until its tip rested against the inner face of the wall opposite the point of entry. The base of the esophagus then jerked spasmodically forward and backward as the contents of the cell—granules and globules—passed inward through the lumen of the spear toward the esophagus.

The diagonal opening at the tip of the spear averaged 1.1 μ in diameter, as measured in 10 mature specimens of the nematode. This agrees rather closely with the size of the opening (1.2 μ) calculated from Thorne and Swanger's (16) illustration of the nematode and may be related to the lower size limits of cells that the nematode can attack successfully. In order to test this hypothesis, 30 representative cells of each host microorganism were measured. Cells of *Chroococcus* sp. ranged from 5 to 8.5 μ, whereas those of *Chlorella vulgaris* and *Tetraedron* sp. varied from 5 to 10 and from 6 to 12 μ, respectively. Cysts of *Drepanomonas* sp. ranged from 14 to 25 μ, and conidia of *Cephalothecium* sp. averaged 12.09 μ. Measurements were not made of the cross-sectional diameter of hyphae of *Cephalothecium* sp., but they are obviously smaller than any of the cells on which the nematode feeds. Repeated observations of unsuccessful attempts by the nematode to feed on hyphae of *Cephalothecium* sp. indicate that its failure to make entrance into a hyphal cell may stem from a combination of 2 factors: 1) the lumen of the cell may be too small to envelop the diagonal opening of the spear, and 2) the cell wall may resist penetration by virtue of its toughness.

The period of feeding on individual cells and the time required to exhaust the cellular contents varied with the particular host microorganism. Feeding was of least duration on spores of the fungus, averaging about 1 second per spore. The spores underwent an instantaneous loss of turgor, collapsing like balloons as soon as the spear was inserted. The protoplasmic contents of the spore were depleted, and the spore membrane lost its visual identity, becoming difficult to see under the microscope. Algal cells were drained of much of their protoplasm and partially collapsed in 3–4 seconds (Fig. 1), whereas cysts of the protozoan were attacked for periods ranging from 20–40 seconds without obvious exhaustion of their contents. Feeding on cysts appeared incomplete, and there was no collapse of the cyst wall. Predaceous feeding on other nematodes endured for periods ranging from 3–5 minutes, until collapse of the body of the host nematode was complete and only the cuticle and cuticularized structures remained.

Distribution of D. ettersbergensis.—The nematode has been recognized several times in the water extractions from soil samples collected in different parts of Louisiana from about the roots of oats. Mixtures of nematodes and associated microorganisms from these soil samples added to Petri plates of 3 per cent water agar usually resulted in the isolation of a few specimens of *D. ettersbergensis*. It seems probable that this small population is maintained and selectively increased by its feeding on the host microorganisms present in such soils. Soil samples that yielded the nematode in this manner included 4 from oats in Ruston fine sand at Homer, La., 1 from oats and 1 from common rye grass in Richland silt loam at Winnsboro, La., and 1 from oats in Miller silty clay loam at Curtis, La. Whereas the significance of such isolations is not at all clear, it is obvious that isolation by culturing is dependent upon the extraction of both the nematode and the host microorganisms upon which it feeds. Oats, and perhaps other grasses, provide the local environmental conditions for the occurrence of a favorable ecological complex of these organisms.

De Man (12), in his original description of the nematode, referred to its abundant occurrence in moist Ettersberg (Germany) earth in July and stated that specimens were sent to him by Dr. Zograff, who collected them from a forest of linden and poplars near the village of Kounzova, Russia. The systematic microscopic examination of nematode samples extracted from soils and the additions of freshly extracted nematode mixtures to algal cultures should reveal a more extensive geographical occurrence of *D. ettersbergensis* than is now known.

Maintenance of D. ettersbergensis on a mixture of host microorganisms.—Five experiments were conducted with Petri plate cultures containing a variety of microbial hosts growing on water agar and maintained in darkness at room temperatures (Table 1). The frequency of occurrence and the abundance of host microorganisms were both extremely variable, but the data illustrate some average populations obtained. A mixed substrate composed of *Chroococcus* sp. and *Drepanomonas* sp., as these occur on the surface of an agar culture, is shown in Figure 2.

Experiment 5 was initiated by placing a single adult of *D. ettersbergensis* on each of 10 agar plates. Survival of nematodes occurred in 4 of 10 plates, and the average increase in population of these plates was more than 3-fold per week for the 19-day period (Table 1).

Primary food preferences of the nematode could not be ascertained by observations on feeding in mixed microbial cultures, since several microorganisms frequently were attacked during an observation period. There was a general tendency, however, for the nematode to feed most frequently on the most abundant host microorganism in a culture.

Comparative population trends on individual microbial hosts.—Two experiments were conducted to determine the relative value of individual host microorganisms in promoting population increase of the nematode. Petri plate cultures containing pure populations of the different host microorganisms were established, and each was inoculated with 3 adult specimens of *D. ettersbergensis*. The number of replicate plates was sufficient to provide 4 cultures for each treatment and sampling during an experiment. Purity of the cultures of microbial hosts was checked repeatedly throughout the experimental periods by systematic microscopic examinations and photomicrographs. Photographs of the different host microorganisms, as they occurred in the cultures, are shown in Figures 4–6. Attempts to maintain high populations of the individual host microorganisms were generally successful, thus assuring an abundance of food for the nematode throughout the course of the experiments.

The results (Fig. 7, 8) demonstrated that *Chroococcus* sp. is the most favorable host, among those tested, for propagation of *D. ettersbergensis*. The green algae (*Chlorella vulgaris* and *Tetraedron* sp.) promoted a rapid and sustained increase in population but were considerably less effective than the blue-green alga. *Drepanomonas* sp. and *Cephalothecium* sp. failed to

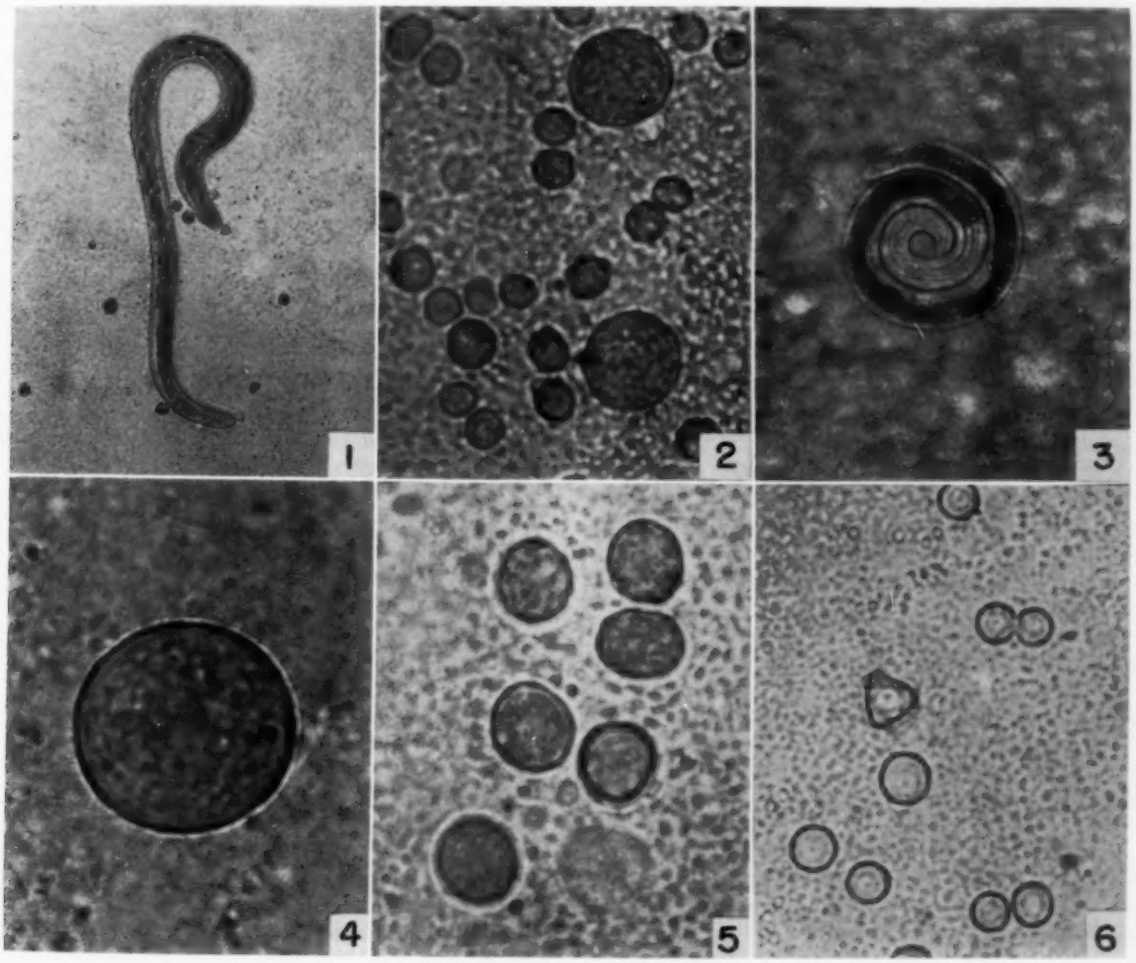

FIG. 1–6. Microorganisms photographed in agar cultures.—Fig. 1. *Dorylaimus ettersbergensis* feeding on a cell of *Chroococcus* sp.—Fig. 2. A mixed microbial substrate for the nematode composed of *Drepanomonas* sp. and *Chroococcus* sp.—Fig. 3. Spiral habit assumed by *Dorylaimus ettersbergensis* in drying agar.—Fig. 4. A single cyst of *Drepanomonas* sp. (enlarged about 1450 ×).—Fig. 5. Cells of *Chroococcus* sp. (enlarged about 1450 ×).—Fig. 6. Cells of *Chlorella vulgaris* and *Tetraedron* sp. (enlarged about 700 ×).

TABLE 1.—*Population increase of Dorylaimus ettersbergensis on mixtures of microbial hosts*[a] *growing on water agar in Petri plates maintained in darkness at room temperatures*

Experiment	Days	No. cultures extracted	Nematodes per culture[b]
1	0		10
	21	12	134
	39	12	68
	49	12	95
	70	12	40
2	0		?
	14	6	213
	27	8	770
	49	4	1561
3	0		3
	21	8	31
	35	8	90
4	0		3
	19	12	43
	30	11	86
	48	12	287
5	0		1
	19	4	35

[a] The microbial hosts were *Cephalothecium* sp., *Drepanomonas* sp., *Chroococcus* sp., *Chlorella vulgaris*, and *Tetraedron* sp.

[b] Figures for 0 days indicate the numbers of nematodes placed on each plate.

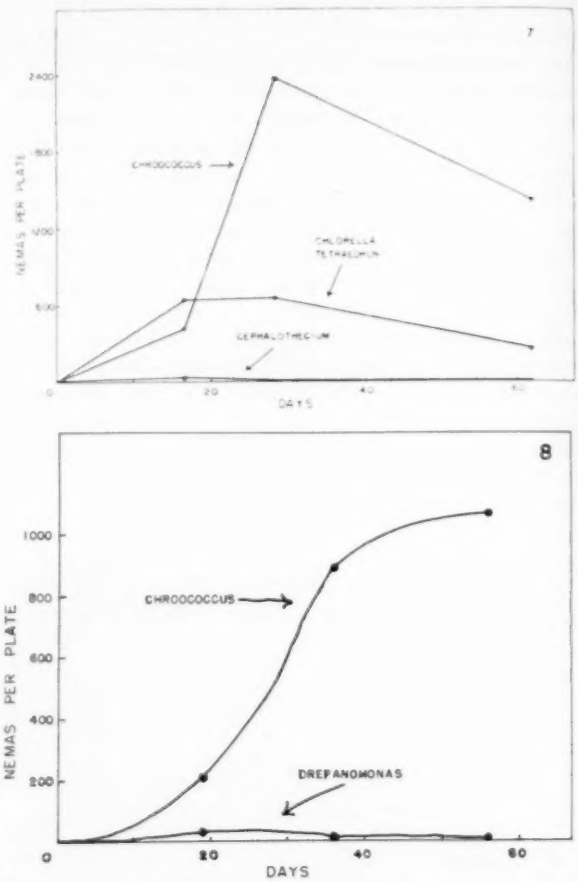

FIG. 7-8. Relative population trends of *Dorylaimus ettersbergensis* cultured on pure populations of individual host microorganisms.—Fig. 7. Comparison of nematode population increase on *Chroococcus* sp. with that on *Chlorella vulgaris* and *Tetraedron* sp. and on *Cephalothecium* sp.—Fig. 8. Comparison of nematode population increase on *Chroococcus* sp. with that on *Drepanomonas* sp.

sustain an initial 10-fold increase in population past the second or third week. The feeding on fungus spores and on protozoan cysts, although intensive in culture, probably provides the nematode supplemental food only. In mixed cultures, the algae apparently are most important in the nutrition of the nematode.

At present, *D. ettersbergensis* is being cultured on *Chroococcus* sp. and on a mixture of *Chroococcus* sp. and *Drepanomonas* sp. in 3 per cent water agar and in a special algal medium (5) rendered semisolid with 3 per cent agar. Populations have been uniformly high, averaging 2 to 5 thousand per Petri plate after 5 or 6 weeks. Yields have been roughly equivalent on the pure and mixed microbial substrates and on the 2 types of media. No noticeable superiority of the algal medium has been demonstrated under the prevailing cultural conditions. Apart from furnishing a supplemental food source, *Drepanomonas* sp. prevents excessive growth of bacteria during the early stages of culture. This appears to facilitate the growth of *Chroococcus* sp. and to raise the final population levels of the nematode. Initial growth of *Chroococcus* sp. in cultures is improved also by heat treating suspensions of cells at 50° C for 10 minutes, just prior to their use as inoculum. This practice, combined with partial disinfestation of the nematode with chemicals, provides the most favorable conditions yet achieved for insuring a rapid population increase on *Chroococcus* sp.

The role of bacteria in the present methods of culture of the nematode.—The feeding activities of the ciliate stage of *Drepanomonas* sp. demonstrate its nutritional dependence upon bacteria in culture. The rapid multiplication of algae on water-agar plates maintained in darkness for long periods, coupled with their lack of green color and the presence of bacteria, suggest a nutritional dependence of the algae upon metabolic products of the bacteria. It has never been experimentally determined how such algae can exist commonly in certain soils, in the absence of light, at depths ranging from 35 to 50 cm (3). Their association with heterotrophic bacteria furnishes a logical explanation of such occurrences (17).

In the present cultural regimen, the maintenance and increase in populations of *D. ettersbergensis* appear to be largely independent of light and darkness and chemical composition of the culture medium. Transfer of partially disinfested nematodes and of Chroococcus cells from nematode-free cultures to nutrient broth revealed that bacteria are associated independently with both organisms. Therefore, elimination of bacteria from cultures of the nematode on *Chroococ-*

cus sp. can be achieved only by the separate disinfestation of both nematode and alga. Culture of the nematode with an alga under bacteria-free conditions will probably necessitate use of an algal medium and exposure of the cultures to light.

Survival of the nematode in desiccated agar.—The typical spiral habit assumed by dorylaims in plant tissues (15) was exhibited by *D. ettersbergensis* in drying agar that retained gelatinous characteristics (Fig. 3). In air-dried agar, survival of the nematode is probably dependent on the presence of the egg stage. In order to test this point, cultures containing abundant nematodes and eggs in a thin agar layer were permitted to dry over a 3-month period so that shrinkage and solidification of the agar resulted. The cultures were observed, found free of the nemic stage, and then incubated 3 additional months in this desiccated condition. Water was then added; 2 weeks later, the excess water was removed and the regelatinized agar searched for the presence of active nemas. Their presence in all cultures demonstrated survival of the nematode in the egg stage for a period of 3 months.

Discussion.—An attack on certain fundamental problems of phytonematology and microbiology awaits methods for the culture of plant parasitic nematodes, particularly in chemically defined media. Progress has been made by Dougherty and his associates (2) toward the axenic culture of free-living rhabditid nematodes, and Mountain (13) has demonstrated the tissue culture of a plant endoparasite. Insofar as the author is aware, however, no serious attempt has been or is being made to ascertain the preliminary steps leading to the axenic culture of a plant parasitic nematode.

Knowledge of feeding mechanisms in different groups of nematodes, resulting from the observations of Linford (8), Linford and Oliveira (9), and Christie and Arndt (1), indicates that relative nutritional nonselectivity and simplicity among nematodes would be most likely to reside in an ectoparasitic type that feeds by the mechanical withdrawal of cellular contents from a host. *D. ettersbergensis* should prove to be an ideal species of plant ectoparasite for attempts at axenic culture because of its wide host range and its habits of feeding by forcible removal of cellular protoplasm.

Recently compiled data on the amino acids of blue-green algae (4, 10, 11) indicate that the proteins of these organisms possess the same amino acid composition as do those of higher organisms. This would support the view that any artificial substrate formulated for *D. ettersbergensis* should provide the amino acids found commonly in the proteins of both primitive and more complex organisms (4).

The principal problem inviting immediate work on culture of plant parasitic nematodes is a technical one, which may be illustrated by a query—Is it possible to culture such a nematode in a continuous menstrum, or must nutrients be furnished through a membrane or in discrete packets resembling microscopic cells, from which the nematode can take nutrients, as under natural conditions?

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INOCULATION, OVERSUMMERING, AND SUSCEPT-PATHOGEN RELATIONSHIP OF SEPTORIA TRITICI ON TRITICUM SPECIES [1]

H. M. Hilu and Wayne M. Bever [2]

SUMMARY

Abundant sporulation of *Septoria tritici* was obtained on Elliot-V8 agar, a synthetic medium supplemented with a proprietary mixture of vegetable juices. Good infection was obtained in small-scale experiments with the "cotton" method of inoculation (applying spores to leaf by means of absorbent cotton previously soaked in a spore suspension). Applying a dilute spore suspension under pressure (water-soaking) to the leaves gave satisfactory infection and a differential varietal reaction without the use of a moist chamber. Roots and crowns were not infected from inoculated seeds nor in artificially infested sterilized soil.

The fungus oversummers in infected volunteer plants and as pycnidia in wheat debris lying on the surface of the soil. High temperature materially decreased the viability of the spores. Since infection was obtained only on species of *Triticum*, the possibility of oversummering on other grass hosts was eliminated.

Under greenhouse conditions, all varieties of *T. vulgare* tested were susceptible, but their reaction varied in the field. Resistance was found in some varieties of *T. durum*. It was expressed as a low percentage of leaf infection and as small numbers and small size of lesions on the inoculated leaves.

The fungus usually penetrated either the opened or closed stomata and grew in all directions between the mesophyll cells. In later stages, many mesophyll cells became completely surrounded, and the hyphae were limited only by the bundle sheaths. In general, the leaf and the cellular structure appeared normal during the incubation period, which ranged from 7 to 16 days and averaged 9 days. It was long at relatively low temperatures and longer in intermediate and resistant varieties than in susceptible ones. The cells of the suscept began to die at the time the symptoms appeared. In later stages, all mesophyll cells in the lesions died, and the hyphae remained intercellular and progressed outside the necrotic area between the living cells. Subepidermal pycnidia, of the symphogenous type, developed in the substomatal chambers and varied in size and shape. They were larger in the susceptible than in the intermediate or resistant varieties. In those cases where infection was established in the intermediate and resistant varieties, the suscept-pathogen relationship was essentially the same as in the susceptible variety. The fungus penetrated the leaves of the 3 varieties equally but developed only rarely in the 2 intermediate and resistant varieties. In addition, the hyphae that developed in these 2 varieties grew relatively slower than that in the susceptible variety, resulting in a longer period of incubation.

When a culture filtrate of the fungus was driven inside the leaves, necrotic lesions similar to those produced by the pathogen appeared on all varieties. The appearance of these lesions corresponded to the time required for the appearance of the symptoms on wheat leaves inoculated with a spore suspension.

Speckled leaf blotch of wheat caused by *Septoria tritici* Rob. ex Desm. is worldwide in distribution. The disease generally is considered of minor importance, although serious losses have been reported in local areas (4, 8, 13).

This study was undertaken to find a suitable method of obtaining abundant spore production in culture, to find an easily applied method of artificial inoculation, to study the suscept-pathogen relationship in susceptible and resistant wheat varieties, and to determine how the pathogen lives through the summer under Illinois conditions.

REVIEW OF LITERATURE.—The cultural characteristics of the pathogen have been studied by many investigators (3, 5, 11, 17, 19). In general, a culture transfer of spores of this fungus produced thin-walled hyaline hyphae and a yeast-like flesh-colored growth consisting mainly of secondary spores. It later produced a coarse olivaceous-to-black mycelium that occurred in dense masses and formed a stroma-like cover over the mucous flesh-colored growth that was unsuitable for artificial inoculation. Pycnidia similar to those in diseased wheat leaves have been reported to occur in culture, but Bremer et al (3) failed to find such pycnidia on a number of standard media.

The same method of artificial inoculation in the field and greenhouse has been used by most investigators. It consists of spraying on the plant, with an atomizer or a hand sprayer, a spore suspension obtained from a pure culture or from infected wheat leaves. The plants are kept moist for 24–120 hours after inoculation. For greenhouse inoculation, Beach (2) and Renfro and Young (14) stroked the leaves between thumb and forefinger that had been dipped in the inoculum. The plants then were placed in a moist chamber for 3–4 days and rewet for 24 hours after 8 days. Beach also

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used a wire loop to spread the spore suspension over the surface of the leaf.

S. tritici oversummers in infested wheat plant residues lying on the surface of the soil and in infected volunteer wheat plants (5, 12, 13, 19). The spores borne in pycnidia may cause infection in the fall when they are exposed aboveground. In contrast, spores lost their viability in 1 month when infected leaves were buried 1½ in. below the surface of the soil.

In addition to species of *Triticum*, *S. tritici* has been reported to infect the following grasses: *Aegilops cylindrica* Host (16), *Brachypodium sylvaticum* (Huds.) Beauv. (7), *Dactylis glomerata* L. (20), *Poa annua* L., *P. secunda* Presl. (17), *P. pratensis* L. (1, 17, 19), and *Secale cereale* L. (1, 18, 19).

The pathological histology of the organism was studied by Weber (19). He found that the fungus penetrated the leaf directly and grew between the epidermal cells; at the appearance of the first symptoms, the fungus already had occupied most of the chlorotic area and had produced wefts of mycelium in the substomatal chamber. The fungus grew in all directions and was limited in growth only by the vascular bundles. Weber found that pycnidia always occupied the substomatal chambers.

Resistance has been reported in many varieties of species of *Triticum*. Luthra et al (12) found, by inoculation tests, that varieties of *T. durum* Desf. grown in Punjab, India, were almost immune, whereas *T. compactum* Host and *T. vulgare* Vill. were susceptible. Beach concluded from inoculation experiments that varieties of *T. vulgare* differed in susceptibility and that the fungus failed to infect *T. compactum*, *T. dicoccum* Schrank, *T. durum*, and *T. polonicum* L.

MATERIALS AND METHODS.—Wheat and other grasses grown in the greenhouse for inoculation with *S. tritici* were seeded in 3-in. pots containing black loamy soil.

The fungus was isolated from pycnidia in wheat leaves collected in different areas of Illinois. The pycnidia were smeared on 2 per cent water agar in Petri plates and incubated at room temperature for 20 hours. Single germinating spores were removed by a needle and transferred to potato-dextrose agar or Elliot-V8 agar (V-8 is the proprietary name of a vegetable juice product of Campbell Soup Company) slants. The isolates were maintained by subculturing alternately by mass transfers and by single-spore transfers every 1 or 2 months. The pathogenicity of all isolates was determined on susceptible C. I. 12557[3] (unnamed *T. vulgare*) wheat seedlings in the greenhouse; a spore suspension in distilled water was sprayed by means of an atomizer on to the leaves after removal of the bloom. The plants were then placed in a moist chamber that was covered with wet burlap sacks, or the inside of the chamber was supplied with a continous fine spray of distilled water from an atomizer. The plants were left in the moist chamber for 72 hours and then removed to a greenhouse bench. All isolates were similar in pathogenicity.

A suitable medium that would support rapid growth and abundant production of spores was sought. Oatmeal, onion, potato-dextrose, prune, Elliot, and Czapek's agar and Richard's solution were prepared according to Riker and Riker (15). Other media such as malt agar and yeast and malt extracts were prepared from Bacto dehydrated media according to directions of the manufacturer. V-8 agar was prepared as described by Diener (5). In the preparation of Elliot-V8, Czapek-V8, and Richard-V8 agars, the filtrate of a 1:5 dilution of V-8 juice was substituted for distilled water. Three replicates of 15-cc slants were used for each medium and were seeded equally with a 6-mm loop of a spore suspension in distilled water. The spores were from a culture derived from a single spore. The cultures were then incubated for 22 days, and a microscopic examination was made before growth was measured by the dry-weight method described by Lilly and Barnett (10).

For artificial inoculations, a spore suspension was obtained by adding 10 ml of distilled water to an Elliot-V8 agar slant. The spores were scraped gently by a transfer needle until about 10–15 spores could be counted in a 430× compound microscope field. The technique of inoculation used for the pathological histology studies was to remove first the bloom and then to apply the spore suspension to the leaves by means of a camel's-hair brush. The plants then were placed in a moist chamber and sprayed with an atomizer until droplets of distilled water could be seen on the surface of the leaves. The plants were left in a moist chamber for 72–96 hours.

Spores from pycnidia in wheat leaves exposed to field and underground environment and to different temperatures were tested for viability. Naturally infected leaves containing abundant pycnidia were collected on May 21, 1955, from the Agronomy South Farm, Urbana, Illinois. In the first test, the leaves were wrapped in a single layer of cheesecloth and hung on a stake at a level 2 ft. above the soil surface for 9 months. In another experiment, the leaves were wrapped in a single layer of cheesecloth or Lumite (a synthetic fiberglass screen, manufactured by Chicopee Mills, Inc., New York) and buried in the soil to a depth of about 3 in. Leaves also were placed in small coin envelopes and stored for 9 months in temperature-control chambers set at 5°, 10°, 15°, 25°, 30°, and 36°C. The percentage of germination was the criterion of the viability of the spores. At monthly intervals, 5–10 pycnidia were obtained from a random sample of leaves, smeared individually on Petri plates containing 2 per cent water agar, and incubated 24 hours at 20°C. The germination of 1200 spores or more was determined. Periodic single-spore isolations were made and used to test the pathogenicity of the spores after exposure to the above conditions.

The pathological histology was studied in whole mounts or in paraffin sections of inoculated wheat

[3] C.I. refers to the accession number of the Cereal Crops Section, U. S. Department of Agriculture.

leaves of the varieties C.I. 12557 (*T. vulgare*), Kubanka (C.I. 2094, *T. durum*), and Arnautka (C.I. 1493, *T. durum*), which are susceptible, intermediate, and resistant, respectively, in their reaction to *S. tritici*. Leaf sections from the inoculated areas were removed at intervals of 12 hours from inoculation until the pycnidia were fully developed. For whole mounts, small sections of the leaf were cleared and stained in a saturated aqueous solution of chloral hydrate containing 0.03 per cent acid fuchsin and 0.02 per cent fast green. They then were heated in a steam sterilizer 5–10 minutes, then rinsed and mounted in lactophenol. The usual paraffin method was used for sectioning leaves (9). The sections were killed and fixed in formalin-aceto-alcohol for 24 hours and dehydrated in the tertiary butyl alcohol series. They then were embedded in paraffin, cut 10–20 μ in thickness with a rotary microtome, and stained in fast green plus safranin. The desired results were obtained when the sections were stained in safranin for 2 hours.

RESULTS.—*Spore production in culture.*—In general, the fungus grew better on media containing plant extracts than on synthetic media. On Elliot-V8, Czapek-V8, and Richard-V8 agars, secondary spores developed by budding off continuously from germinating spores, thus producing a pinkish to flesh-colored growth. The stroma-like mat of dark thick-walled hyphae failed to develop on these media, and growth consisted mostly of spores. The fungus grew rapidly and abundantly on all 3 media, but it grew best on Elliot-V8 agar.

Inoculation experiments.—A number of methods of inoculation, in which spore suspensions of the pathogen were either applied to the surface of the leaves or injected into the leaf or culm of the wheat seedlings, were tried in the greenhouse. After the bloom had been removed, the inoculum was applied to the surface of the leaves by means of an atomizer, a camel's-hair brush, or a piece of absorbent cotton. With the exception of those inoculated with the atomizer, the plants were sprayed with distilled water after inoculation until droplets could be seen on the surface of the leaves. The inoculated plants first were placed in a moist chamber 72–96 hours and then removed to a greenhouse bench. Application of inoculum to the leaf surface by the fingers (2, 14) also was tried. Inoculation was most successful when the inoculum was applied with a camel's-hair brush and least successful when applied with an atomizer. Satisfactory results were obtained when the inoculum was applied to the leaf with a piece of cotton or with the fingers, but the former method is more applicable for small inoculation experiments in the greenhouse.

An inoculation technique not requiring a moist chamber would increase the efficiency and rapidity of inoculation. Good infection was obtained when inoculum was driven (under air pressure) inside the leaf by an atomizer or by a syringe supplied with a 1-hole rubber stopper in place of the needle. In both cases, the spore suspension spread between the mesophyll cells and produced a water-soaked area around the point of application. Inoculum injected into the culm of the wheat seedling by means of a hypodermic needle gave unsatisfactory results. Necrosis, with an abundance of pycnidia in the dead tissues, occurred 7–8 days after inoculation. A differential reaction among wheat varieties was not obtained by this method when a concentrated spore suspension was used. Therefore, this method should be used only with a dilute spore suspension (8–10 spores in a 100× microscope field). When the inoculum was injected into the culm of the wheat seedling, necrotic lesions containing pycnidia occurred on the developing leaves around the puncture of the needle. These lesions were more abundant and larger on C.I. 12557 than on either Kubanka or Arnautka. This method, however, cannot be used successfully because lesions occur only on leaf growth developing after inoculation.

Under some conditions, *Septoria tritici* has been reported to cause a crown rot in winter wheat in late fall or early spring (6). Several crown- and root-inoculation methods were used in the greenhouse to study this phase of the disease. Only negative results were obtained.

Oversummering stage.—The oversummering stage, as referred to in this paper, extends from harvest to the initial appearance of the disease in the fall. It is important because it serves as the source of primary inoculum for fall infection, and subsequently has some influence on the amount of spring infection. As mentioned earlier, it has been reported (19) that *S. tritici* survives the summer months as pycnidia in infested wheat debris lying on the surface of the soil or in infected volunteer wheat plants. When pycnidia in wheat leaves were placed 2 ft. above the soil and exposed to natural environment from May to February in the present tests, the initial spore germination of 97 per cent was maintained through June and July, but germination decreased to 47 per cent in August and continued to decrease gradually thereafter. By February, only 6 per cent of the spores germinated. In October, the majority of pycnidia were empty, and many spores had lost their protoplasm. Abundant rainfall, which occured early in October, evidently caused the liberation of the spores. It is concluded that such liberated spores provide ample inoculum for inciting the disease in the fall.

When infected leaves were buried in the soil, all of the leaves were rotted and most of the pycnidia had disappeared in 1 month. The small number of pycnidia that were found proved to be empty.

The germination of spores borne in pycnidia in wheat leaves stored at different temperatures agreed in part with results of a similar experiment reported by Luthra et al (11). In both experiments, the spores lost their viability rapidly at temperatures of 15°–30°C, the loss in viability being complete after 2 months. At temperatures of 5°–15°, the spores retained their vi-

ability for the duration of the experiments, and the percentage of germination was highest at the lowest temperatures used. At the lower temperatures (5°–10°), however, the percentage of germination reported by Luthra et al was lower than that obtained at corresponding temperatures in the present study.

In order to explore the possibility that the fungus might oversummer on some other host, a number of reported suscepts and other available grasses, namely *Aegilops cylindrica*, *Agropyron trochycaulum* (Link) Malte, *Agrostis alba* L., *Avena sativa* L., *Brachypodium sylvaticum*, *Dactylis glomerata*, *Poa pratensis*, *P. secunda*, *Secale cereale*, *Triticum vulgare*, *T. durum*, and *T. monococcum*, were inoculated in the greenhouse with *S. tritici*. Inoculations included both the application of the spore suspension to the surface and the driving of inoculum into the leaves. Only species of *Triticum* were infected; however, necrotic areas developed at the point of application when the spore suspension was driven into the leaves in some of the grasses, but neither infection nor pycnidia developed in these areas.

Pathological histology.—A number of wheat varieties of *T. vulgare* and of *T. durum* were artificially inoculated in the greenhouse in order to select varieties representing susceptible, intermediate, and resistant types. All wheat varieties were inoculated in the seedling stage. All varieties of *T. vulgare* tested were extremely susceptible. Even Wisconsin Pedigree No. 2 and RedChief, which have been reported highly resistant, were severely infected. The varieties of *T. durum* ranged in reaction from susceptible to resistant, but a majority of them were highly resistant but not immune. C.I. 12557, Kubanka, and Arnautka were selected as representative varieties showing susceptible, intermediate, and resistant reactions, respectively. The results of the inoculation of these varieties indicated that resistance to this disease is due in part to a low probability of the pathogen becoming established in the leaf after penetration. In the few cases in which infection took place in the intermediate and resistant leaves, hyphal growth was relatively slow. Table 1 shows the difference in reaction of the 3 representative varieties to *S. tritici* when plants of these varieties were inoculated in the greenhouse. The lesions that developed on the resistant varieties were small. Inconsistency in infection and the length of the incubation period also were expressions of resistance. The incubation period ranged from 7 to 16 days in the susceptible varieties, with an average of about 9 days. It tended to be longer at low than at high temperatures, confirming the results obtained by Weber (19). Regardless of temperature, however, the incubation period was 4–10 days longer in the resistant and intermediate than in the susceptible varieties.

The suscept-pathogen relationships were studied in detail in inoculated seedlings of the susceptible, intermediate, and resistant wheats. In the susceptible variety, the spores began to germinate by producing small germ tubes at either end or at the septa, approximately 12 hours after inoculation. Penetration, as a rule, was through the stoma (Fig. 1,A) contrary to another report (19). Occasionally, however, penetration was through the cuticle. Stomatal penetration was first observed 24 hours after inoculation; germ tubes were not necessarily attracted to the stomata, as some grew over the stomatal pore. The germ tubes were about 1 μ in diameter and penetrated either the opened or closed stomata. Following penetration, the hyphae always enlarged slightly and grew between the cells of the suscept in any direction; they branched either at a septum or at the middle of the hyphae cell. The hyphae first grew very slowly, but some hyphae traversed the entire thickness of the leaf within 100 hours after inoculation. The majority of the hyphae grew lengthwise of the leaf, but others, the branches in particular, grew obliquely or crosswise. In some cases, the hyphae grew between the epidermal and the first mesophyll cell layer. They grew very intimately with the cell walls and branched between the cells. In others, the hyphae grew intercellularly between other subepidermal layers, either parallel to the surface of the leaf or at various angles.

TABLE 1.—*Reaction to Septoria tritici of susceptible, intermediate, and resistant wheat seedlings when inoculated in the greenhouse by the "brush" and "cotton" methods*[a]

Variety	No. of leaves inoculated	Percentage of leaves showing infection	Average No. of lesions per leaf
C.I. 12557 (susceptible)	51	84.3	9.0
Kubanka (intermediate)	40	15.0	1.1
Arnautka (resistant)	43	6.9	0.32

[a] Results of the 2 methods were combined.

During the first 9 days after inoculation, the fungus traversed the leaf many times and progressed extensively in all directions. Macroscopically, the leaves appeared healthy, and the presence of the fungus inside the leaf could be detected only by microscopic examination. Nearly all cells were normal, with only an occasional dead or slightly plasmolyzed cell. The hyphae conformed first to the configurations of the intercellular spaces, and growth was restricted only by the vascular tissues. The sclerenchyma cells of the bundle sheath appeared to protect the vascular bundles from fungus attack. The fungus, however, was not completely restricted to the areas between the vascular bundles but spread into new areas of the leaf by growing between the mesophyll cells around the vascular bundles. Occasionally, anastomosis was observed between 2 hyphae that either separated immediately after fusion or grew side by side for a short distance between the cells of the suscept. At the end of this 9-day period, the hyphae had enlarged considerably and were approximately 2.5 μ wide, had many vacuoles, and were unevenly septate. At this time, the

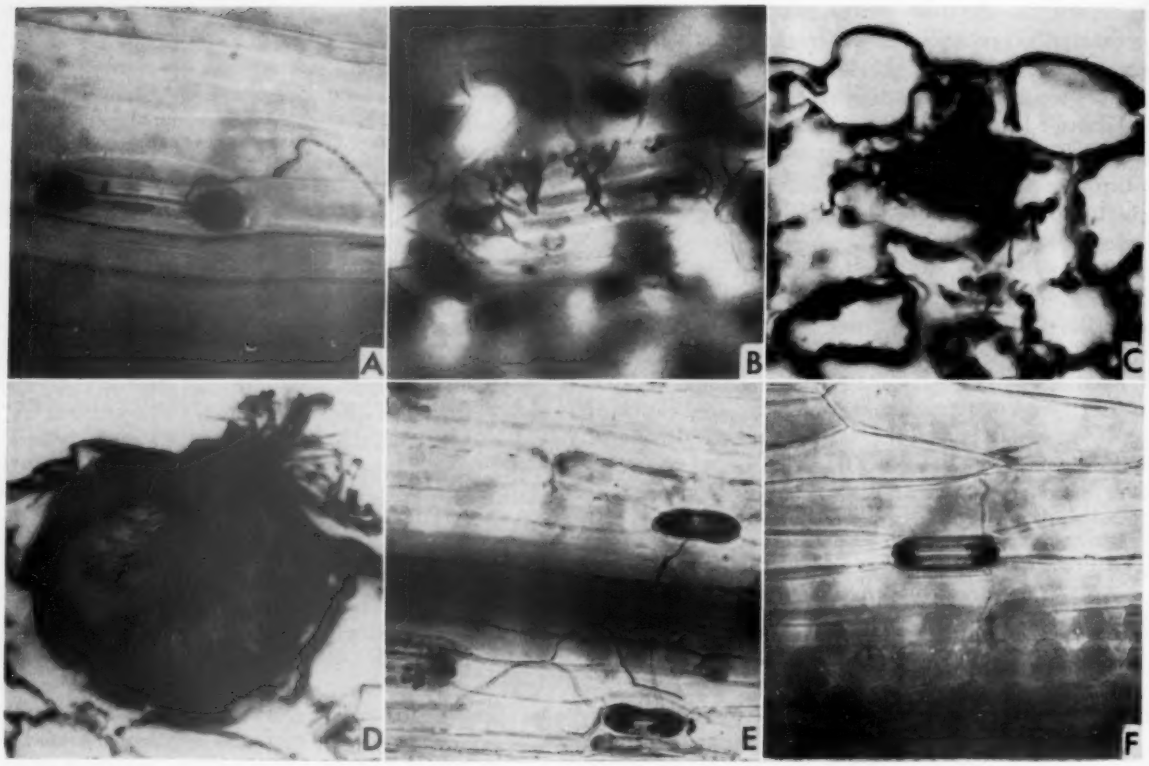

FIG. 1. A) Stomatal penetration (×450). B) Initiation of a pycnidium, showing the side under a stoma (×450). C) Cross section of the pycnidial primordium, showing the pointed beak (×450). D) Cross section of a mature pycnidium (×450). E, F) Hyphal growth, 100 hours after inoculation, in leaves of C.I. 12557 and Arnautka, respectively (×270).

fungus started its asexual reproductive stage. One or 2 hyphae, growing under the epidermis, completely surrounded the stoma along the outer walls of the subsidiary cells. Usually 1 or more hyphae grew under the stoma and fused at 2 points with this hyphal loop. All hyphae then branched extensively, and the branches, which were produced side by side (Fig. 1.B), appeared first as small buds that later elongated and fused with other branches. The initial wall of the pycnidium was formed after extensive hyphal growth and fusion; the pycnidial primordium appeared later as a globular mass of hyphae with a pointed beak growing toward the stomatal pore (Fig. 1.C). Pycnidial development, therefore, is of the symphogenous type, originating from a number of branches that were produced by different hyphae. Macroscopic symptoms appeared suddenly 11 days after inoculation. In certain areas, 5–6 cells were killed, and the hyphae, for the most part, were still intercellular. Here, without exception, pycnidia in various stages of development were observed under the stomata. Individual pycnidia continued to form for a long period. The pycnidial primordium, which consisted of a globular base and a pointed beak, enlarged in diameter to conform to the shape of the substomatal chamber (Fig. 1.D). Hence, the beak, which was first long and narrow, became wide and lost its identity. The ostiole developed at the apex and conformed to the shape of the stoma. Upon maturity, the spores were exuded usually in a cirrus through the stomatal pore only. The peridia of the pycnidia varied in width from 3 to 5 layers of pseudoparenchymatous cells. The inner layer usually produced the conidiophores as small buds that later became fusoid and pointed at the tip. At times, they were curved and approximately 6–8μ in length and 1.5–2μ in width. The spores were borne on the tip of the conidiophores, at first as small buds that later elongated and became filiform and septate upon maturity. Microspores, a second type of spore often observed in pycnidia collected from naturally infected wheat leaves, were not evident in the histological specimens studied.

In the later stages of the disease, nearly all mesophyll cells in the necrotic lesions were killed. The chloroplasts usually disappeared, and the cell contents became homogenous. Some cells were separated from each other and consisted merely of distorted walls. In general, the vascular bundles were intact except in rare cases in which the hyphal growth was extremely abundant. Most hyphae remained intercellular and spread a considerable distance beyond the necrotic lesions. Few hyphae were observed inside either the living or dead cells of the host. After death of the cells had occurred, the walls were surrounded so intimately that both the walls and hyphae, which stained identically, appeared as a single structure.

In those cases where infection did occur in the inter-

mediate and resistant varieties, the suscept-pathogen relationship was similar to that in the susceptible variety; however, during and after the initiation of infection, the hyphal growth was much slower in the intermediate and resistant leaves than in the susceptible. One hundred hours after inoculation, the hyphae had branched extensively and traversed a dozen cells in C.I. 12557, whereas they had traversed only 3 or 4 cells and remained unbranched in Arnautka (Fig. 1,E,F). The slow rate of growth in Arnautka and Kubanka undoubtedly accounted for the long period of incubation, as described earlier. Growth was slow in these 2 varieties until the appearance of symptoms; at this stage, the amount of hyphal growth per unit area of killed tissue was approximately the same in all 3 varieties, and in each variety the hyphae had grown beyond the necrotic lesions. In the susceptible variety, however, more and larger lesions developed than in the resistant ones, and the hyphae therefore had spread to a greater extent between the living cells. The fungus sporulated in all 3 varieties. The size and number of pycnidia per unit area of killed tissue from different sources or from the same variety varied (Table 2). The pycnidia were largest in diameter in the susceptible variety and smallest in the resistant variety. Pycnidia developing under natural conditions were less abundant per unit area of killed leaf tissue but were larger than those developing in the 3 varieties tested in the greenhouse.

The germination of the spores on the leaf surface of the resistant, intermediate, and susceptible varieties was similar. The difference in the number of stomata in the susceptible and resistant varieties was not statistically significant, thus the opportunity for penetration was about equal. The number of penetrations and the number of hyphae that successfully developed after penetration in the leaves of the 3 varieties were determined. Potted seedlings of the 3 varieties were inoculated by the "brush" method in the greenhouse. The pots were placed in a moist chamber for 96 hours and then removed to a greenhouse bench. Seventy-two and 100 hours after inoculation, 14 sections of leaves, approximately 2 cm long and 0.6 cm wide, were selected at random from each variety. They were cleared, stained, and mounted in lactophenol. The area of each leaf-section was examined for penetration in the 72-hour samples and for fungal growth in the 100-hour samples. Approximately the same number of hyphae penetrated the leaves of each of the 3 varieties 72 hours after inoculation; however, a larger number of hyphae became established in the susceptible than in either the intermediate or resistant varieties (Table 3). It was concluded, therefore, that the small number of lesions, the inconsistent infection, and the low percentage of leaf infection in the resistant and intermediate varieties were caused by the inability of the hyphae to become established inside the leaves after penetration.

TABLE 2.—*Size and number of pycnidia in leaf lesions of susceptible, intermediate, and resistant greenhouse-grown wheat seedlings, 1 month after inoculation with Septoria tritici, and of pycnidia developed under natural conditions*

Source	Range in diameter [a] (μ)	Average diameter [a] (μ)	No. of pycnidia in 100 microscope fields [b]
C.I. 12557 (susceptible)	81.4-185.0	139.5	803
Kubanka (intermediate)	81.4-174.0	113.0	848
Arnautka (resistant)	74.0-251.8	130.9	794
Natural infection	74.0-248.1	147.8	646

[a] Based on 100 pycnidia.
[b] Based on 100 microscope fields, each 0.63 mm.

TABLE 3.—*Number of stomatal penetrations and establishment of Septoria tritici, 72 and 100 hours after inoculation, in susceptible, intermediate, and resistant wheat seedlings*

Variety	No. of leaf sections	No. of stomata penetrated after 72 hours	Percentage of hyphal establishment after 100 hours
C.I. 12557 (susceptible)	14	19	72
Kubanka (intermediate)	14	18	15
Arnautka (resistant)	14	21	4

Toxin studies.—The effect of the metabolic by-products produced by the pathogen in liquid media when applied to the leaves of wheat seedlings was studied. The fungus was grown for 25 days at room temperature in 250-ml Erlenmeyer flasks containing 50 ml of sterilized Bremer malt soup,[4] 200 ppm, in charcoal-treated distilled water. Controls consisted of the nonseeded medium and of sterile charcoal-treated distilled water. After the 25-day period, the sterile and seeded media were filtered through No. 1 filter paper in a Buchner funnel and through a sterilized Seitz filter. Each filtrate was driven inside the leaves of potted C.I. 12557, Kubanka, and Arnautka seedlings by means of a syringe supplied with a 1-hole rubber stopper. Leaves of the 3 varieties were inoculated with a spore suspension of the fungus in a like manner. The leaves in all treatments remained normal until 7 days after inoculation, when necrotic lesions developed on the leaves of the 3 varieties treated with the culture filtrate of the fungus and on those inoculated with the spore suspension. The necrotic lesions were similar on the 2 sets of plants, except that those on inoculated plants contained many pycnidia. In contrast, the leaves treated with sterile water or with the filtrate of the nonseeded medium remained healthy, except for an occasional minute dead area resulting from mechanical injury. This evidence indicates, therefore, that some metabolic

[4] Nondiastatic malt extract neutralized with potassium carbonate. (Manufactured by Malt Extract Co., 217 North Walcott Ave., Chicago, Ill.)

by-product of the fungus in culture produced necrotic lesions similar to those incited by the pathogen on the leaves of the 3 varieties tested. Furthermore, the time required for the appearance of these lesions corresponded to the time required for the appearance of the symptoms on the inoculated leaves.

Discussion.—One of the important problems in working with *S. tritici* has been the lack of success in producing an abundance of spores on artificial media. Spores are necessary to obtain successful artificial inoculations. On potato-dextrose agar, this fungus produces mats of closely packed hyphae with relatively few spores; consequently, it is of little value for the production of inoculum. In contrast to this, it was found that Elliott-V8 agar was an excellent substrate for the production of an abundance of secondary spores with a minimum of hyphal development.

Another factor that has made it difficult to study resistance to Septoria, particularly under field conditions, has been the necessity of supplying a high humidity over a long period of time after inoculation. Under most field conditions, this is difficult to accomplish. A technique was devised whereby the incubation chamber was eliminated. Inoculum was driven under pressure inside the leaf by means of an atomizer or with a syringe supplied with a 1-hole rubber stopper. Excellent infection was secured by this technique; however, it is severe and a dilute spore suspension must be used to obtain satisfactory differential varietal reactions.

S. tritici survives the summer months in Illinois in infected volunteer wheat plants and as pycnidia in wheat leaves and leaf sheaths that had been previously infected during the spring. These infested parts must lie on or above the soil in order for the spores to retain their viability and pathogenicity during the summer and early fall. When so located, they may be subjected to high temperature, which was found to decrease the viability of the spores more than any other environmental condition. The possibility that other hosts might carry the fungus through the summer is very unlikely, because infection failed to develop in any grass tested other than species of *Triticum*.

Resistance in species of *Triticum* to *S. tritici* appears to be due in part to erratic and infrequent establishment of infection after penetration, and in part to slow growth of the fungus in those cases where infection is established. The slow rate of hyphal growth would explain the long incubation period in such varieties. The cells of the suscept did not exhibit any morphological response in limiting the hyphal ingress beyond penetration. Thus, the actual reaction that limits fungus growth after penetration in the resistant and intermediate varieties remains unknown. This barrier did not always function, for a small number of hyphae overcame this barrier, and growth progressed between the cells of both the intermediate and resistant leaves. The hyphae first grew slowly and later thrived and became as abundant in the lesions of such varieties as in lesions of the susceptible variety. In addition, they sporulated and the hyphae advanced beyond the dying cells. Pycnidial development was similar in the 3 kinds of varieties; however, the pycnidia in intermediate and resistant varieties were smaller in size than those in the susceptible varieties. It can be concluded, therefore, that the resistance manifested by the durum varieties is only partial and consists of limiting the ingress of most hyphae after penetration.

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STRAINS OF THE BARLEY YELLOW-DWARF VIRUS [1]

Thomas C. Allen, Jr. [2]

SUMMARY

Upon inoculation of 31 cereal varieties with barley yellow-dwarf isolates, varietal differences in symptom expression were noted. Four of the 31 cereal varieties were then selected as differential test hosts for all virus isolates. The varieties chosen were the barley varieties Black Hulless, Rojo, and Atlas 46 and the oat variety, Coast Black. Forty-three isolates of yellow-dwarf virus were tested. When these isolates were transferred to the various plant species, 16 strains were distinguished. Each test species was inoculated with each isolate 2–9 times. The ability of the virus to cause stunting and discoloration on the hosts was used to differentiate strains; the differences were statistically significant at the 1 per cent level. The strains were grouped for convenience into 7 types based on their ability or inability to cause discoloration in each host. Two of the types thus separated included 10 of the 16 strains. It was found that mixtures of these strains could be produced by simultaneous inoculations and that such mixtures could be separated by aphid transfer to different hosts. No evidence of natural mixtures was found in the 16 strains described. Also noted was the absence of positive cross-protection reactions between strains.

The barley yellow-dwarf virus is widely distributed (1). Since it has been described so recently (10), only a limited amount of research has been done to determine the presence of strains of the virus. A review of work concerning strains was undertaken with particular reference to any basis by which possible strains of the barley yellow-dwarf virus could be separated.

Strains of aphid-transmitted yellows-type viruses have been separated by Clinch and Loughnane (5) and by Webb et al (16). Clinch and Loughnane (5) isolated a mild yellows and etch yellows strain of the sugar beet yellows virus. Strain separation was based on the symptoms expressed by the hosts. The mild yellows strain did not protect against the etch yellows strain.

Webb et al (16) separated 4 strains of the potato leaf roll virus from a collection of 56 isolates. This separation was based on symptoms produced on 1 host, *Physalis floridana* Rydb.

Several reports have been made on possible strains of barley yellow-dwarf virus. In 1955, Bruehl and Toko (4) described a Washington strain that differed from the original virus described by Oswald and Houston (10, 11) in symptom expression on different cereal hosts. Takeshita (14) reported 2 strains of the virus that were separated by differences in virulence on greenhouse-grown plants. One caused severe symptoms whereas the other induced mild symptoms in all hosts tested. A similar separation was observed earlier by Oswald and Houston (unpublished data). The research reported herein presents a more detailed picture of strains of the barley yellow-dwarf virus. An abstract on the problem has been presented (1).

MATERIALS AND METHODS.—Sterilized soil placed in 6-in. pots and watered with distilled water was used in all investigations. The use of distilled water was necessary to avoid damage caused by the excessive salts present in Davis tap water. The soil consisted of 3 parts Yolo sandy loam and 1 part sand. Seeds were generally untreated and planted ½ in. deep. The plants were watered daily, and a small amount of nitrogen in the form of ammonium sulfate was added once every 3 weeks; the sulfate was used partially to counteract the extreme alkalinity of the soil.

The temperature in the greenhouse ranged from 10° to 38°C, depending on the time of day and time of year. During most of the year, however, it was possible to maintain a temperature of approximately 21°C. The light intensity ranged up to 13,000 foot-candles in the summer. Low light intensity in the winter apparently suppressed symptom expression—symptoms being absent in many cases. To remedy this, a bank of fluorescent lights was hung 3 ft. above the pot tops. These provided an intensity of 500 foot-candles in the center of the bench and one of 300 foot-candles at the corners; these supplemented the natural light to give an intensity of 500 to 600 foot-candles on the cloudiest winter days and proved to be sufficient for good symptom expression. The artificial photoperiod was set at 12 hours, with the lights on from 6 a.m. to 6 p.m. The relative humidity of the greenhouse averaged approximately 70 per cent.

Since the apple-grain aphid, *Rhopalosiphum prunifoliae* (Fitch), had previously been shown to be an efficient vector of the virus (11) and is comparatively easy to handle, it was used exclusively as the vector during this investigation. Use of this single aphid species avoided possible variation due to differences in the ability of different species to transmit the virus strains (15). As reported by Oswald and Houston (11) and confirmed by Freitag (unpublished data), the virus is persistent in the vector. Freitag also found that this aphid acquired the virus from infected plants in a feeding period of 5 minutes and that infective aphids could transmit it to healthy seedlings in a 5-minute test feeding. The aphids remained viruliferous for 8 serial daily transfers to healthy plants, retaining

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the virus as long as they lived.

Nonviruliferous apple-grain aphids were obtained as described by Oswald and Houston (10). These were maintained on Sacramento barley thickly sown in 6-in. pots. A typical cage used for rearing aphids is shown in Figure 1.A. A lining of cotton between the wooden base of the large cheesecloth cage and the pot insured confinement of the aphids and prevented the entrance of any foreign aphids. As soon as live plants showing yellow-dwarf symptoms were received, recovery trials were made by transferring nonviruliferous aphids to the plants by use of a small camel's-hair brush. Cheesecloth cages were kept over the plants during the aphid feeding period. After they had fed on the plants for 1–7 days, the aphids were transferred to healthy cereal seedlings that were in the 1- or 2-leaf stage. Ten aphids were placed on each of 3 seedlings in a 6-in. pot (Fig. 1.B); each lot included nymphs of the various instars, as well as apterous and alate adults. This made the best use of all aphids in a colony and avoided the chance exclusive use of a developmental stage that might be a less efficient vector than some others; such differences have been reported by various workers (3, 7, 13).

Individual-plant cages were made of glass tubing $\frac{3}{4}$ in. in diameter and 8 in. long. The top of the tubing was covered with a layer of cheesecloth held in place by a rubber band. The tube was placed over the plant with the base in contact with the soil and was supported with a pot label inserted in the soil and held to the tube with rubber bands (Fig. 1.B). Aphid transfers were made in the headhouse in subdued light at a temperature of about 21°C. The aphids were allowed to feed on the plants in the headhouse so that rapid changes in light or temperature would not result in moisture condensation in the cages or on the plants. After the aphids had fed for 24 hours, the cages were removed and a 1 per cent parathion spray was used to kill the aphids. After being visually checked for live aphids, the inoculated seedlings were placed under the lights in the greenhouse.

Whenever viruliferous aphids were fed on seedlings, nonviruliferous aphids were fed in exactly the same way on other seedlings of the same varieties; these served as controls. For each experiment, nonviruliferous aphids were placed on at least 3 seedlings of each variety being tested.

Host plants in which various isolates were maintained were handled as follows. Plants inoculated with the various isolates were placed in a separate greenhouse section and maintained for future use as sources. These plants were sprayed frequently with 1 per cent parathion and fumigated weekly. A reisolation was made possible by washing the parathion from these plants before placing aphids on them. There were no fluorescent lights over these older plants, and the plants were watered with tap water.

Height measurements and the degree of discoloration were first recorded 10–14 days after seedling inoculation. The height recorded was the average height of the 3 plants in a pot when leaves were held vertically. Subsequent readings were taken every 3–4 days for 4–10 weeks after inoculation.

After preliminary tests, a system of symptom ratings based on a combination of discoloration and stunting was devised. The ratings consisted of the following categories: HR—highly resistant, with no discoloration and no stunting; R—resistant, with discoloration but no stunting; T—tolerant, with discoloration and slight stunting (1–24 per cent); S—susceptible, with discoloration and moderate stunting (25–49 per cent); VS—very susceptible, with discoloration and severe stunting (50 per cent or more). Severity of discoloration was often directly proportional to the amount of stunting. Later, a need for modification of this rating system was demonstrated. This will be discussed later.

In a few cases, the fresh weight of the tops was obtained after sufficient height measurements and notes on discoloration were taken. When weights were used, all the plants in an experiment were cut in the morning while the soil in the pot was still damp, eliminating any noticeable effect of water loss that might occur later in the day.

Each virus isolate received was assigned a code consisting of 3 parts. The first part is a capital letter indicating the host from which the virus was recovered, i.e. *B* for barley, *O* for oats, *W* for wheat, *R* for rye, and *G* for a grass host. The second part, a number, indicates the county from which the isolate was obtained; the county from which the first isolate was obtained received a 1, and following counties were numbered consecutively. The third part, a lower case letter, indicates the order in which each isolate was received from a particular county, i.e. the first isolate made from any county was designated *a*, the second *b*, etc. As an example, the code name *B-7-a* denotes a virus isolate from a barley plant from the seventh county from which isolates were received and that it was the first plant obtained from that particular county.

The code name of isolates received from outside California also consists of 3 parts. The state is indicated first by its abbreviation. The second part, a number, shows which isolate from that state was involved, and the last letter of the code refers to the host from which the virus isolate was recovered. An isolate designated Wis-1-O would have been obtained from Wisconsin, would be the first isolate from the state, and would have been recovered from oats.

The possibility of the occurrence of natural mixtures of virus strains was studied by attempting to separate strains from naturally and experimentally inoculated individual plants. In the experimentally inoculated plants, 2 strains were introduced into 1 plant by means of viruliferous aphids.

Two methods were used to separate these mixtures of strains. In one, nonviruliferous aphids were allowed to multiply on the plants inoculated simultaneously with the 2 virus strains. Ten of these aphids were transferred to each of the 3 seedlings of a variety. If any of the 3 seedlings varied from the others in symp-

tomatology, these variants were chosen for subinoculation into the 4 cereal host species. It was assumed that strains could eventually be separated by a continuation of this inoculation cycle.

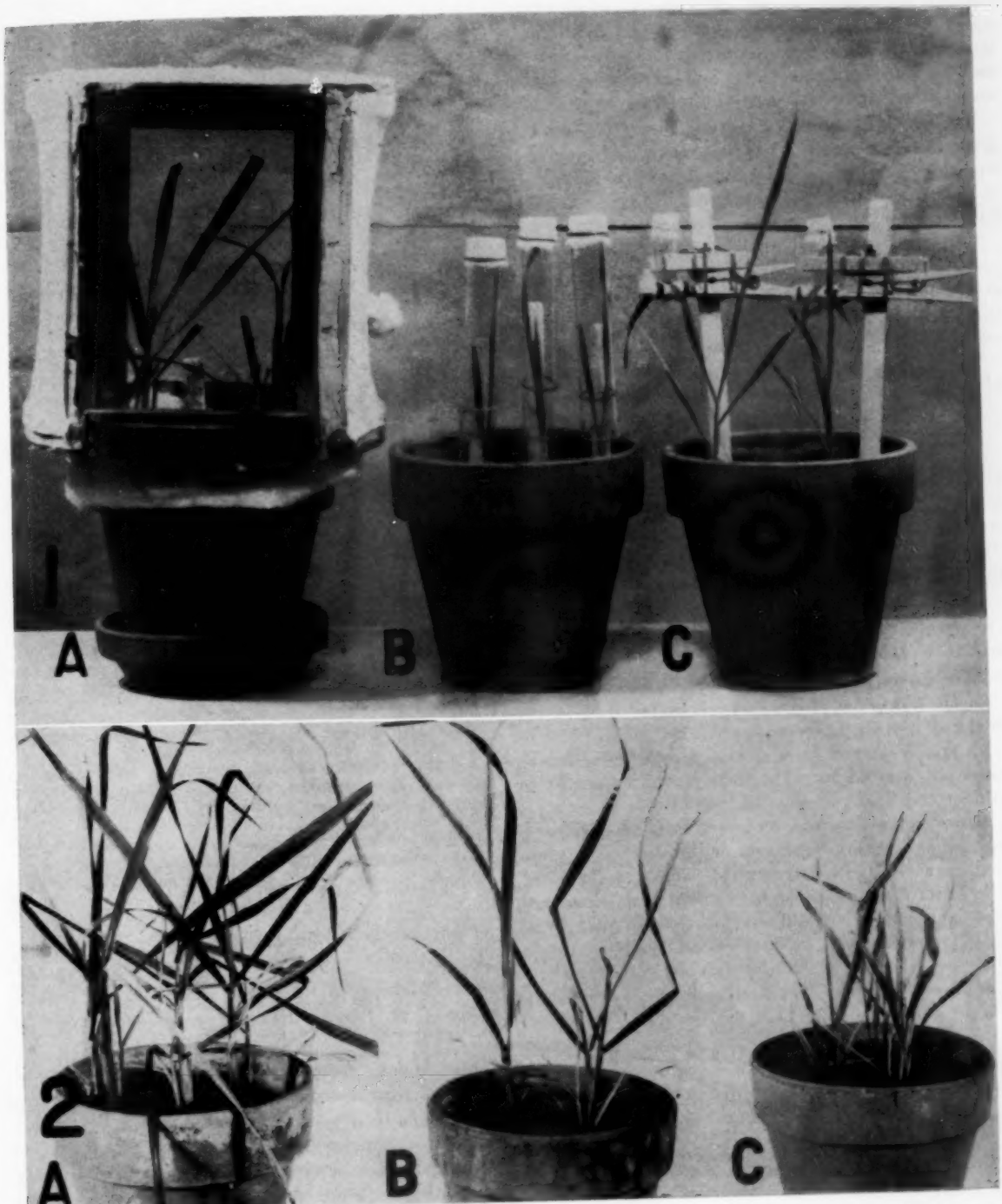

FIG. 1–2. Fig. 1. Cages used in aphid-transmission studies of barley yellow-dwarf virus. A) Large cheesecloth cage used for aphid maintenance and virus recovery from older plants. B) Test tube cages used to transfer the virus to seedlings. These cages insured confinement of the 10 aphids on each seedling. C) Leaf cages that confined 1–10 aphids on portion of leaves.—Fig. 2. The effect of isolate O-3-a of barley yellow-dwarf virus on 3 oat varieties. A) Bond oats. Leaves are discolored, but plants are only slightly stunted. B) Coast Black oats. Leaves are serrated, distorted, and discolored, and plants are moderately stunted. C) Abegweit oats, showing leaf discoloration and serration, severe stunting, and death of the youngest leaf.

The second method consisted of transferring single aphids to each test plant of the 4 host varieties after the aphids had fed on plants containing the mixture. Results were checked by allowing nonviruliferous aphids to multiply on the test plant and then transferring 10 aphids each to 12 other plants—the 4 species used as differential hosts. It was assumed that this would effect a mass transfer of the strain or strains.

RESULTS.—*Symptoms of yellow dwarf.*—Thirty-one different cereal varieties were inoculated with the various isolates received. This range included 13 varieties of barley, 11 of oats, 6 of wheat, and 1 of rye (Table 1). Later 1 grass species was inoculated.

With few exceptions, the symptoms expressed by various hosts in response to inoculations with barley yellow-dwarf virus were similar to those described by Oswald and Houston (11, 12). Black Hulless barley reacted as described, but in addition demonstrated a purple coloring that replaced the yellowing of leaf edges; this purpling seemed to be accentuated by cool temperatures. The barley variety Abate showed no yellowing, but purpling appeared in blotches and then progressed down from the tip of the older leaves as does the yellowing in other varieties. Apparently, purpling instead of yellowing occurs where anthocyanin is the dominant pigment instead of carotene.

Instead of yellowing or purpling, an orange to red discoloration developed on oats; cool weather favored the deeper color. Only such varieties as Bond showed a bright red color, whereas most varieties demonstrated various shades of orange. Leaf serrations were evident in some oat varieties.

Barley yellow-dwarf virus was recovered from inoculated rye that showed no apparent symptoms. Isolate B-1-b was recovered as long as 6 months after inoculation into Merced rye. The virus proved to be just as virulent as when it was maintained in oats or barley.

Although the virus was recovered from other grasses, only 1 grass species, *Danthonia californica* Boland, was used as a host in this research. This grass showed yellow-dwarf symptoms when received, but it had not been included in Oswald and Houston's host range (12). Symptoms on *D. californica* consisted of purpling of leaves from the tip downward. Symptoms on the various other species again were well described by Oswald and Houston (12).

Symptoms incited by different isolates.—The varietal reactions of 3 of the isolates are shown in Table 1. The results indicate that there are distinct differences in a variety's reaction to the different isolates of barley yellow-dwarf virus.

Strikingly different symptoms were incited by any one isolate on the different varieties. The effects of isolate O-3-a are an example of the variation of symptoms that one isolate can incite in different oat varieties (Fig. 2). Coast Black oats showed some leaf serration and distortion accompanied by an orange discoloration, but the plants were not severely stunted (Fig. 2,A). Bond oats were not stunted appreciably (Fig. 2,B), but an orange to red discoloration was induced. When inoculated into Abegweit oats, this isolate produced severe stunting of the plants, discoloration and serration of leaves, and death of the youngest leaf (Fig. 2,C).

TABLE 1.—*Reaction of 31 cereal varieties to 3 isolates of barley yellow-dwarf virus*

Variety	C.I. No.[a]	Reaction[b] to indicated isolate		
		O-1-d	O-3-a	O-4-a
Barley				
Abate		T	HR	
Atlas	4118	S		
Atlas 46	7323	R	R	T
Black Hulless	666	T	S	HR
California Mariout	1455		R	
Club Mariout	261	S	T	
Hannchen	531	T	R	
Nakona Wase	754	R	T	
Rojo	5401	T	HR	HR
Vaughn	1367	T	R	
	1227	T	HR	
	1237	T	HR	HR
	2376	T	HR	
Oat				
Abegweit	4970	T	VS	T
Arlington	4657	T		
Bond	2733	R	R	R
Branch	5013	S	R	R
California Red	1026	T	T	T
Clinton	3971	S	T	S
Coast Black	1025	T	S	R
Kanota	839	S	R	R
Ped 7	1154	R	R	R
Traveler	5661	R		
Ventura	3989	T	T	T
Wheat				
Baart	1697	T	T	S
Baart 46	12386	S	T	
Harvest Queen	5314	T	T	S
Ramona 50		T	T	T
Sonora 37	11902	T	T	T
White Federation 38	11906	T	T	T
Rye				
Merced		HR	HR	HR

[a] C.I. refers to the accession number of the Cereal Crops Section, U. S. Department of Agriculture.

[b] Rating according to original symptom rating method as follows: HR—highly resistant, no stunting and no discoloration; R—resistant, no stunting but discoloration present; T—tolerant, stunted up to 25 per cent and discolored; S—susceptible, stunted from 25 to 50 per cent and discolored; VS—very susceptible, stunted over 49 per cent and discolored.

One isolate, G-9-c, was recovered from California oatgrass, then inoculated back into healthy oatgrass from Black Hulless barley. Leaf purpling developed 3 weeks after this inoculation. The virus was then again recovered in an apparently unmodified form from the grass. It was found that 4 randomly selected isolates, O-3-a, Wis-2-O, O-10-b, and B-10-a, incited similar symptoms in the oatgrass. Isolate O-7-b was the only one used that failed to produce symptoms in California oatgrass.

Selection of a differential host range.—The next logical step was to choose a host range that was small

enough to allow testing of all the isolates received but one that separated a maximum number of isolates. From results such as those shown in Table 1 and from reference to Oswald and Houston (11), 3 barley varieties and 1 oat variety were chosen for the differential host range upon which all isolates would be tested. Black Hulless barley was chosen because it was observed to be very susceptible by Oswald and Houston (11) and it was stunted to different degrees by various isolates in the preliminary inoculations. Atlas 46 was chosen primarily because its brilliant yellow symptom response is very easy to distinguish. Rojo barley was chosen because its reactions to the isolates were different from those of Black Hulless and were similar to those of a number of varieties previously observed to be resistant in the field. Coast Black oats was chosen because it showed more variability of reaction to various isolates than did other oats. It also produced strong coloring in response to all isolates used in this survey period.

The 4 cereal varieties chosen for the differential host range were inoculated with each of the isolates maintained or received after this host range was established. When 2 or more virus isolates induced the same combination of symptoms in the various test hosts, the isolates were considered to be of the same strain. Of a total of 43 isolates studied, 16 strains were separated on this basis.

Strain differentiation by a modified symptom rating system based on discoloration and extent of stunting.—Results of many repeated experiments showed the need for a modification of the original rating system in order to differentiate the 16 symptom combinations or strains. Although discoloration seemed to remain constant for each isolate in the repeated trials, the percentage of stunting often varied over a considerable range. This range was plotted for each isolate on all 4 varieties (Fig. 3). Little stunting was caused by the various isolates on Rojo barley. Different isolates are not labeled on the graphs, since only the over-all picture was desired.

The graph representing the responses of Black Hulless barley demonstrates fairly good separation of strains on the basis of the amount of stunting caused by the isolates. With this host, responses can be classed as tolerant, susceptible, and very susceptible. Classification of the stunting incited by the isolates on Coast Black oats cannot be made, since the range of stunting induced by any isolate was too great; 1 isolate incited no stunting in 1 trial and incited up to 60 per cent stunting in subsequent trials. The only response of Atlas 46 that can be definitely classified is a very susceptible one. The reactions of Rojo barley were classified as resistant, tolerant, or susceptible.

The validity of a rating system based on stunting was tested in further experiments involving a Latin square design. Aphids were used to inoculate 4 barley seedlings with an isolate producing either HR, T, S, or VS reactions. Nonviruliferous aphids were fed on a fifth seedling, which served as a control. Differences between the heights of plants infected by the different isolates were not all statistically significant at the 1 per cent level; however, plant height was based on the average of 3 plants in a single pot. Therefore, 3 plants in each of five 6-in. pots were inoculated in a second similar experiment. One month after inoculation, final height readings were taken. The LSD at the 1 per cent level was equivalent to about 10 per cent of the height

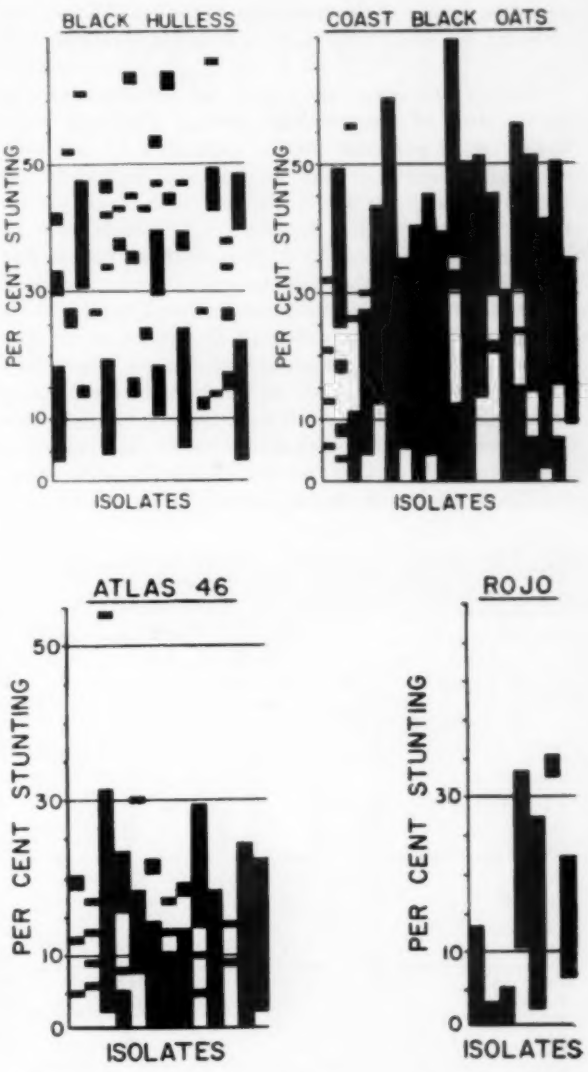

FIG. 3. Graphs demonstrating percentage stunting incited by the 43 virus isolates on Black Hulless barley, Coast Black oats, Atlas 46 barley, and Rojo barley. Each black square or column represents the range of stunting incited by that particular virus isolate. Responses of Black Hulless barley fall within the tolerant, susceptible, and very susceptible classifications. Responses cannot be classified as to stunting incited on Coast Black oats, since the range of stunting is too great. The only response of Atlas 46 barley that is different from those of other isolates is that caused by the isolate that produced 55 per cent stunting. Rojo barley was not stunted more than a susceptible reaction, but tolerant and resistant reactions can be distinguished.

of the control plant. In this particular test, each of the 4 groups differed significantly from the others in plant height.

Similar tests with only 3 plants (in a 6-in. pot) were made with the other varieties in the differential host range. Statistically significant differences were obtained with Rojo barley. With Atlas 46 barley, only those plants inoculated with 1 virus isolate (B-5-c) differed significantly from the others on the basis of stunting. With Coast Black oats, the row and column variation exceeded the treatment variation. These tests revealed the same results as seen on the graphs (Fig. 3).

These facts were taken into consideration in the modification of the original system. Divisions were drawn at 10 per cent, 30 per cent, and 50 per cent stunting. Plants showing no pigment change and a height within 10 per cent of the control were designated highly resistant (HR). Resistant (R) plants were in the same height range as highly resistant plants, but were discolored. The tolerant (T) group included plants that were discolored and stunted from 11 to 30 per cent, whereas plants showing discoloration and 31–50 per cent stunting were designated as susceptible (S). Plants with severe discoloration, leaf edge serration, dead growing points, and stunting of more than 50 per cent were classified as very susceptible (VS).

In tests with various isolates (Table 2), the ratings for Black Hulless barley were HR, T, S, and VS; for Rojo barley, the ratings recorded were HR, T, R, and S. HR and VS (with 1 isolate) were the only specific ratings recorded on Atlas 46 barley; intermediate ratings were represented by a plus sign, which indicated the presence of discoloration. Since stunting was variable in Coast Black oats, only discoloration was recorded with this host; HR was used to designate the absence of discoloration, whereas the plus sign again indicated the presence of discoloration.

The several isolates tested were classified into 16 strains, based on the absence or presence of discoloration and the extent of stunting of the 4 test varieties (Table 2). These 16 strains were divided into 7 types, depending upon whether or not discoloration was induced in the 4 varieties. The strain types were coded on the basis of presence (+) or absence (0) of discoloration in Black Hulless barley, Rojo barley, Atlas 46 barley, and Coast Black oats, respectively. Thus, ++++ designated strains (Type I) that discolored all 4 varieties, +0++ designated strains (Type II) that discolored all but Rojo barley, +00+ designated strains (Type III) that discolored only Coast Black oats and Black Hulless barley, ++0+ designated strains (Type IV) that discolored all but Atlas 46 barley, 00++ designated strains (Type V) that discolored only Black Hulless and Rojo barley, 00+0 designated strains (Type VI) that discolored only Atlas 46 barley, and 000+ designated strains (Type VII) that discolored only Coast Black oats.

TABLE 2.—*Reaction of 4 cereal varieties to 43 isolates of barley yellow-dwarf virus, with a classification of the strains into 16 strains in 7 strain types*

Strain type [a]	Strain	Reaction [b] of indicated varieties: Black Hulless barley	Rojo barley	Atlas 46 barley	Coast Black oats	Isolates [c]
Type I (++++)	1	VS	R	+	+	B-1-b, B-5-d, Ariz-3-O, B-7-a
	2	S	S	+	+	O-12-a
	3	S	T	+	+	B-8-a, O-10-b
	4	S	R	+	+	B-9-a, B-10-d, Ore-1-G, Wash-3-O
	5	T	R	+	+	R-2-a, Md-1-B, Ill-2-O
	6	T	T	+	+	O-1-d
Type II (+0++)	7	VS	HR	VS	+	B-5-c
	8	VS	HR	+	+	G-9-c
	9	S	HR	+	+	B-1-a, B-5-e, O-7-b, B-10-a, B-15-a, B-15-b, B-16-b, Wis-2-O, Ore-1-W, Ark-1-O, Md-2-O
	10	T	HR	+	+	B-1-c, O-3-a, B-5-a, B-11-b, B-11-c, Minn-1-O
Type III (+00+)	11	S	HR	HR	+	O-16-a, Ariz-2-O
	12	T	HR	HR	+	Ill-1-O, Ariz-1-W
Type IV (++0+)	13	T	R	HR	+	O-11-a, Wash-2-G
Type V (00++)	14	HR	HR	+	+	O-4-a
Type VI (00+0)	15	HR	HR	+	HR	G-11-d
Type VII (000+)	16	HR	HR	HR	+	Ill-3-O

[a] Strain types coded according to presence (+) or absence (−) of discoloration on Black Hulless barley, Rojo barley, Atlas 46 barley, and Coast Black oats, respectively

[b] Rated according to modified rating as follows: HR—highly resistant, no discoloration, 0–10 per cent stunting; R—resistant, discolored, 0–10 per cent stunting; T—tolerant, discolored, 11–30 per cent stunting; S—susceptible, discolored, 31–50 per cent stunting; VS—very susceptible, discolored, over 50 per cent stunting; and + indicates discoloration, irrespective of stunting. Each rating is based on 6–27 plants.

[c] Isolates coded according to origin as described in text.

TABLE 3.—*Fresh weight and height of healthy plants of 4 cereal varieties and of comparable plants 2 months following inoculation with each of 2 isolates of barley yellow-dwarf virus* [a]

Variety of test plant	Isolate of virus	Discoloration	Height		Average fresh weight in g			Fresh weight as percentage of control		
			Average (in.)	Percentage of control	Tops	Roots	Total	Tops	Roots	Total
Black Hulless barley	None	—	19		12	6	18			
	Wis-1-O	+	20	105	9	5	14	75	83	78
	B-1-b	+	7	37	2	1	3	17	17	17
Rojo barley	None	—	20		15	12	27			
	Wis-1-O	+	21	105	9	5	14	60	42	52
	B-1-b	—	20	100	11	8	19	73	67	70
Coast Black oats	None	—	17		10	8	18			
	Wis-1-O	+	21	124	8	3	11	80	38	61
	B-1-b	+	12	71	2	1	3	20	13	17
Kanota oats	None	—	19		14	7	21			
	Wis-1-O	+	20	105	7	3	10	50	43	48
	B-1-b	+	19	100	10	5	15	71	71	71

[a] Each figure is the average for 3 plants grown in a 6-in. pot in the greenhouse.

Comparative fresh weight as a basis of strain differentiation.—In the experiments described in the previous section, a number of infected plants that showed definite symptoms of yellow dwarf were taller than the noninfected control plants. It appeared possible, however, that fresh weight of the infected plants might have been less than that of the controls. Consequently, the effects of 2 isolates, Wis-1-O and B-1-b, on the fresh weight of Black Hulless barley, Rojo barley, Coast Black oats, and Kanota oats were determined (Table 3). Data were taken 2 months after inoculation. In each case, the fresh weight of 3 infected plants was compared with that of a like member of healthy controls. Differences between infected plants and healthy ones and those between plants infected with different isolates of the virus were greater and more consistent when comparisons were based on fresh weights than when based on heights. Differences among the fresh weights of roots were greater than those among top weights or among total fresh weights.

Further experiments were effected with various isolates of barley yellow-dwarf virus on barley, oat, and wheat varieties. In all these experiments, only the height and fresh weight of the tops were recorded, since tops are easiest to remove and weigh. In these experiments, Abate barley was rated as tolerant to Wis-1-O and O-1-d on the basis of both height and discoloration (Table 4). Differentiation between these 2 isolates might have been possible on the basis of effects on fresh weight of tops had more plants been tested; however, the in-treatment variation of a given isolate was found to be larger than the difference between the 2 isolates. Results with wheat were quite variable; infected plants surpassed the controls in weight in several cases but in no case did they surpass the controls in height. In the fresh-weight trials with oats, controls were surpassed by infected plants in height and in weight. In the 2 trials with Coast Black oats (Tables 3, 4), the controls were the same height but were much different in weight. The effect of infection by Wis-1-O on the height of plants also differed in the 2 trials. These results do not favor either height or weight as a basis for strain characterization, nor did the results of later experiments in which Latin square experimental designs were used.

Although promising at first glance, fresh-weight readings later demonstrated the same biological variability as did height measurements under the environment provided. Since it is easier to read height than fresh weight and since height measurements permit the keep-

TABLE 4.—*Fresh weight and height of tops of healthy plants of 13 cereal varieties and of comparable plants 1 month following inoculation with various isolates of barley yellow-dwarf virus* [a]

Variety	Average height in in. of plants infected with indicated isolate				Average fresh weight in g of tops of plants infected with indicated isolate			
	None	Wis-1-O	O-1-d	B-7-a	None	Wis-1-O	O-1-d	B-7-a
Barley								
Abate	22	18	21		10	5	8	
Wheat								
Baart	28		24	21	6		7	7
White Federation 38	25		22	21	9		9	6
Sonora 37	19		18	17	7		5	6
Ramona 50	20		19	18	5		5	3
Harvest Queen	16		15	12	10		6	4
Oats								
Abegweit	20	18	17		(15)	6	4	
Coast Black	17	19	18		5	6	4	
Ped 7	23	26	25		7	7	5	
Ventura	24	23	20		6	5	3	
Bond	18	19	19		9	4	4	
Branch	21	22	19		6	7	3	
Clinton	24	22	19		10	7	3	

[a] Each figure is the average for 3 plants grown in a 6-in. pot in the greenhouse, except that figure in parenthesis is for only 1 plant. All plants except controls were discolored.

ing of plants for maintenance of isolates, height and discoloration readings were the basis upon which all isolates were differentiated. The experiments in this section did demonstrate, however, that infected plants can be taller than the control and still have less fresh weight.

Attempts at cross protection.—Since it is not uncommon for 2 different viruses to have overlapping host ranges or to produce similar symptoms (8), positive cross-protection reactions would help to prove a definite relationship among the proposed strains of the barley yellow-dwarf virus. The possibilities of such a reaction occurring between virus strains transmitted exclusively by insects was studied in 1955 by Kunkel (9). He cited evidence of cross protection found by other workers with maize streak virus and with cacao swollen shoot virus. Kunkel demonstrated cross protection between California aster yellows virus and aster yellows virus. Two of these cases involved leafhopper vectors; cacao swollen shoot virus, however, is transmitted by a mealy bug. Although barley yellow-dwarf virus is aphid transmitted, it is a yellows-type virus as is aster yellows virus. This cross protection found between 2 strains of aster yellows virus was the first report of reciprocal cross protection between strains of a leafhopper-transmitted yellows-type virus (9). The cross protection occurred in vectors as well as plants. Another virus, curly-top, which is leafhopper transmitted but not a yellows-type virus, showed no cross protection between strains (6).

Before the above-mentioned report by Kunkel (9) appeared, several different methods were tried by the writer to determine whether or not strains of yellow-dwarf virus will cross protect. In all these attempts, plants infected with 1 virus strain were inoculated with a second as soon as symptoms appeared (1–2 weeks). One such test is summarized in Table 5. In this particular case, measurements on height of singly and doubly inoculated plants provided no information on whether or not there was any cross protection. Data on discoloration, however, showed that isolate B-5-a did not protect against isolate O-1-d. However, B-5-a demonstrated symptoms on Black Hulless barley and Coast Black oats proving the virus was transferred by the aphids. Plants inoculated with O-1-d became discolored regardless of whether or not they were infected with the other isolate. This was true regardless of whether the interval between the 2 inoculations was 1 week or 2 weeks.

TABLE 5.—*Discoloration and height measurements of Abate barley plants that were noninoculated or inoculated (by means of aphids) with 2 isolates of barley yellow-dwarf virus, either alone or with inoculation with one isolate preceding that with the other* [a]

Isolate used for inoculation on date indicated			Data taken 8 weeks after planting [a]	
January 26	February 2	February 9	Discoloration	Height
None	None	None	—, —	23, 24
None	None	O-1-d	+	24
None	O-1-d	None	+	20
O-1-d	None	None	+	19
O-1-d	None	O-1-d	+	21
None	None	B-5-a	—	24
B-5-a	None	None	—, —	20, 22
B-5-a	None	B-5-a	—	22
O-1-d	None	B-5-a	+	22
B-5-a	O-1-d	None	+	21
B-5-a	None	O-1-d	+, +	21, 21

[a] Planted January 18, 1955. Each datum is for a group of 15 plants in five 6-in. pots.

Later, experiments similar to those of Kunkel (9) were attempted. Kunkel waited 6 weeks after symptoms appeared in response to inoculation with the first strain before inoculation with the second strain. This proved too long in this experiment, since yellow-dwarf symptom expression is very dependent upon the age of the plant when it is inoculated (11). Cross-protection results were impossible to record when old cereal plants were used; therefore, the second inoculation was made 2 weeks after symptoms of the first inoculation appeared. Again, no symptom differentiation was apparent. Information might have been obtained by recovery of the virus from the inoculated plants; unfortunately, this was not attempted.

Next, the possibility of a cross-protection reaction in the aphid vector was investigated. Three colonies of 30 nonviruliferous young aphids were confined in 3 pots of healthy Coast Black oat plants. One pot of plants infected with Strain 10 and 1 pot of plants infected with Strain 13 received a similar colony. Two weeks later, 1 colony from the healthy plants was transferred to 3 healthy Coast Black oat plants, another to 3 oat plants infected with Strain 10, and another to 3 oat plants infected with Strain 13. At the same time, the colony from plants infected with Strain 10 was transferred to plants infected with Strain 13. The reverse of this was also effected. After a 2-week feeding on these plants, each colony was transferred to the differential host range. The aphids fed on healthy plants carried no virus, those fed on a source of Strain 10 or 13 transmitted only their respective source strain, and those fed on both combinations of Strains 10 and 13 transmitted a mixture of the 2 strains. Aphid recovery of the strains 1 month later demonstrated that the mixture of Strains 10 and 13 remained in the plants. Since these 2 aphid colonies each transmitted both strains, the acquisition of one strain 2 weeks before the second apparently did not affect the retention of the second. It was, therefore, assumed that cross protection between at least 2 strains of barley yellow-dwarf virus did not occur in the apple-grain aphid.

Tests for mixtures of strains and separation of artificial mixtures.—The question arose as to whether or not some of the isolates were mixtures of several strains of the virus. That this was not the case is suggested by the fact that each isolate had been maintained a long time and transferred numerous times in the greenhouse without apparent change in the symptoms induced. Of the various types, Type I (Table 2) strains were considered the most suitable for experimentation, since all

varieties in the differential host range are susceptible to these strains.

Since these Type I strains remained constant when transferred by 10 aphids, strain separation was attempted with single aphids. Each aphid was fed 24 hours on an infected Coast Black oat plant, then was transferred subsequently to the 4 test hosts; the whole transfer process required 4 days. Each aphid was allowed to feed 24 hours on Black Hulless barley, 24 hours on Rojo barley, 24 hours on Atlas 46 barley, and 24 hours on Coast Black oats, in that order. Many of the aphids failed to transmit virus to any of the plants, some transmitted it to a few, and others transmitted it to all 4 hosts when Strains 2, 3, or 4 were used. When only a few of the 4 test plants developed symptoms, subsequent transfers from these diseased plants were made with 10 aphids per plant to see if a strain separation was effected. In each case of subinoculation, all 4 varieties inoculated showed symptoms typical of those induced by each of the Type I strains. Thus, no separation of at least 3 of the proposed strains was effected, but the experiment did show that aphids did not always transfer the barley yellow-dwarf virus to each host upon which they fed.

Since no natural mixtures were found, artificial mixtures were created and attempts made to separate them. In the first such experiment, Strains 10 and 14 (Table 2) were inoculated simultaneously into Coast Black oats by means of aphids fed on sources of each strain. Seven months later, nonviruliferous aphids were allowed to colonize on this plant and then transferred serially to the 4 test hosts. Symptoms on these plants indicated that only Strain 14 was recovered. Similar results were obtained with single-aphid transfers.

Black Hulless barley seedlings were inoculated separately and simultaneously with Strains 6 and 9. Ten aphids carrying each strain were used. Inoculations with each strain singly resulted in symptoms typical of those usually induced by the respective strain. Two of the 3 plants inoculated with the mixture of the 2 strains showed the tolerant reactions characteristic of infection by Strain 6, whereas 1 showed no symptoms. Recovery was attempted from all 3 of these plants. Only Strain 6 was isolated from the plants that had shown a tolerant reaction; typical symptoms were induced on 2 Black Hulless barley seedlings and none on a third. When recovery was attempted from the plant that showed no symptoms in the original inoculation, one of the 3 plants subinoculated developed symptoms typical of those induced by Strain 9. Apparently, some of the original inoculations resulted in infection by one strain and some the other.

The third artificial mixture consisted of Strains 1 and 14. These were inoculated simultaneously into Black Hulless barley by means of aphids previously fed on each strain source. These plants showed symptoms characteristic of a very susceptible reaction. Nonviruliferous aphids were transferred to these 3 plants and allowed to multiply. After feeding several days on these infected plants, the aphids were transferred to the 4 differential hosts. Symptoms typical of those induced by Strain 1 only developed on 2 plants of Black Hulless barley, on all 3 plants of Rojo barley, and on 2 plants of Atlas 46 barley. The Coast Black oats became discolored, a symptom which could have been induced by either strain. One plant each of Black Hulless and Atlas 46 barley was symptomless. Transfers were made from the symptomless Black Hulless plant. Only Strain 14 was recovered.

Transfers also were made from one of the discolored Coast Black oat plants to Rojo and Atlas 46 barley seedlings. All 3 plants of the former developed symptoms characteristic of infection by Strain 14, whereas one Atlas 46 plant was symptomless and the other 2 were discolored. Recovery attempts from the symptomless plants, both Rojo and Atlas 46, resulted in isolation of Strain 14 only. Transfers from a discolored Atlas 46 plant resulted in the recovery of only Strain 1. Thus, the strains could be recovered from a mixture, depending upon which plants were chosen for sources of the virus.

Discussion.—With most viruses that have been studied intensively, strains have been encountered. The barley yellow-dwarf virus proves no exception to this. The problem, however, was not in finding the strains, but in adequately distinguishing among the strains obtained. The methods that can be used to separate strains of barley yellow-dwarf virus are somewhat limited, since this virus is not mechanically transmitted, no local-lesion host has been found, and the virus itself has not been purified.

Strain differentiation has been achieved in many cases by use of differential symptom expression on common hosts. This method seemed the most logical to use in the present work, especially since differential symptoms incited by the various isolates were noted early in this investigation. While this investigation was in progress, Takeshita (14) separated 2 strains of barley yellow-dwarf virus by means of an oat host range. One strain was virulent and 1 mild on all hosts used. These 2 strains were included in the present work (I11-1-O and I11-2-O).

When attempting to reproduce the results reported in this paper, one should note that all experiments were carried out in the greenhouse; the symptoms noted were not as severe as the field symptoms described by Oswald and Houston (11).

The use of the symptom ratings separated the 43 isolates into 16 symptom combinations, many of the isolates from the rest of the United States being the same as California isolates. The evidence indicated that these symptom combinations were constant and differed enough to be strains. Further clarification of the picture was achieved by grouping the strains into 7 types that might serve as a more useful tool for plant breeders. Since divisions are made on the presence or absence of symptoms, simple experiments can

distinguish between types of strains; plant breeders can easily note the highly resistant hosts because they show no symptoms.

The variability of stunting by any one isolate could be due to the presence of a mixture of strains. The action of such mixtures should be known in order to determine the reliability of the proposed strain differentiation. The facts that no evidence of mixtures was obtained and that some artificial mixtures could be separated are not conclusive evidence that strain mixtures do not exist. Tests were not made with all possible combinations; also, tests with still other hosts might effect separation. If virus-inoculated plants did not show symptoms, they were discarded in some of the earlier experiments. This actually may have resulted in the loss of some strains whose presence was unknown; hence some purification of the strains used may have been effected.

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PURIFICATION AND PROPERTIES OF HYDRANGEA RINGSPOT VIRUS [1]

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SUMMARY

Hydrangea ringspot virus was purified by a combination of ammonium sulfate precipitation and differential centrifugation. The purified preparations consisted of 2 electrophoretic components. The fast-moving component had an ascending mobility of -3.9×10^{-5}cm^2/volt/second at pH 7.0, contained rod-shaped particles, and was infectious. The slow-moving component had a descending mobility of -1.7×10^{-5}cm^2/volt/second, contained no rods, and was noninfectious. A mean particle width of 16.3 mμ and particle lengths of 44 mμ to various greater lengths were determined from electronmicrographs. Infectious preparations gave ultraviolet absorption maxima between 260 and 265 mμ. The infectivity titer, optical density at the absorption maximum, and protein concentration of infectious preparations were correlated.

Hydrangea ringspot virus, recently described by Brierley (2) and Brierley and Lorentz (3), appeared to be stable enough for physical and chemical study. Commercial hydrangeas furnished a ready source of this virus, and virus-free seedlings from seed-transmission trials (3) served as comparable noninfected material. The present investigations were undertaken to characterize the hydrangea ringspot virus and to determine its possible relationships to other known viruses.

MATERIALS AND METHODS.—*Sources of virus.*—Leaves of naturally infected florists' hydrangea (*Hydrangea macrophylla* (Thunb.) DC. var. Merveille) were used as the source of the virus. Since hydrangea ringspot virus was found in all commercial varieties tested and the virus was not transmissible through seed (3), plants grown from seed were used as controls. Leaves of the healthy plants were at approximately the same stage of development as those of the infected plants.

The virus was purified also from local-lesion-bearing leaves of *Gomphrena globosa* L. 8 days after inoculation with juice expressed from infected hydrangea leaves.

Virus assay.—*Gomphrena globosa* was used as a local-lesion assay plant for estimating relative infectivity of various fractions during purification. The leaves were dusted with 320-mesh Carborundum and rubbed with a cheesecloth pad saturated with inoculum. After inoculation, the leaves were rinsed with tap water, and local-lesion counts were made after 7–9 days of incubation in a greenhouse.

Purification.—Preliminary experiments indicated that the butanol-chloroform mixture used by Steere (10) in the purification of tobacco ringspot virus caused considerable loss in infectivity of the hydrangea ringspot virus. Further tests indicated that the virus could be concentrated initially with ammonium sulfate of 0.3–0.6 saturation, by ⅓ volume of ethanol, or by adjusting the pH of the juice to 3.5. The most concentrated preparations were obtained with ammonium sulfate of 0.3–0.4 saturation. Precipitation of the virus with ammonium sulfate, therefore, was adopted as a preliminary step to subsequent differential centrifugation in the purification procedure.

The following purification procedure was employed for both infected and control plant materials. Fresh leaves were harvested in the early morning, weighed, chilled to 4°C, and passed through a hand meat grinder into a container immersed in an ice-water bath. The juice was expressed by manually squeezing through 2 layers of 90-grade cheesecloth and collected in a container immersed in an ice-water bath. The grinding and extraction were repeated on the pressed pulp. Such operations usually yielded 35–40 ml of crude extract from 100 g of infected or healthy leaves.

The crude extract was clarified at 7500G for 15 minutes in a refrigerated Spinco Model L ultracentrifuge. The residue was resuspended in 0.005 M phosphate buffer of pH 7.0 equivalent in volume to 25 per cent of the original extract and centrifuged at 7500G for 15 minutes. Twenty-six g of ammonium sulfate was added to each 100 ml of the combined supernatant and washings. After standing at 4°C for 2 hours, the precipitate was sedimented at 7500G for 5 minutes, and the sediment was washed with phosphate buffer by resuspension and centrifugation at 7500G for 15 minutes. Three such washings were usually made. It was necessary to wash the sediment several times, since tests indicated that a considerable amount of virus would be lost in unwashed sediments. The supernatant from clarification and the washings were pooled and then centrifuged at 84,000G for 5 minutes. The resulting sediment was washed twice with phosphate buffer by resuspension and centrifugation at 84,000G for 5 minutes. This step was intended to remove plant components that were somewhat larger than the virus but that were not removed by low-speed centrifugation. The supernatant and washings were combined, and the virus was pel-

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leted at 144,000G for 30 minutes. The pellet, after resuspension, was then subjected further to 5 additional cycles of ultracentrifugation 34,000G for 5 minutes, washing of sediment, and 144,000G for 30 minutes. The final virus pellet was resuspended by gently shaking with water and the resulting suspension diluted with an equal volume of 0.01 M phosphate buffer. Final clarification was made by acceleration up to 84,000G followed immediately by deceleration.

All protein concentrations were determined by the micro-Kjeldahal method involving direct Nesslerization, a 1:1 H_2SO_4 digestion mixture, and the Nessler reagent of Koch and McMeekein (5, p. 1230). Optical densities of the Nesslerized solutions and ammonium sulfate standards were determined with a Fischer electrophotometer. The factor 6.25 was used as a protein conversion factor for the nitrogen values obtained.

Electrophoresis.—A purified virus preparation containing 7.14 mg of protein/ml was examined in an Aminco-Stern electrophoresis apparatus equipped with the Thovert-Philpot-Svensson optical system. A semimicro clinical cell of 2 × 15-mm cross section was employed. The sample was dialyzed overnight at 4°C against 100 volumes of 0.05 M Na_2HPO_4-NaH_2PO_4 buffer of pH 7.0 in an Aminco mechanical dialyzer (9). Electrophoresis was carried out at a temperature of 1.6°C and at a potential gradient of 5.3 volts/cm. Ascending and descending moving boundaries were photographed at the end of 3 and 4 hours. After 4 hours, visible peaks in the ascending and descending boundaries were fractionated with a syringe fitted with a 12-in. 18-gauge needle. A purified preparation of the control extract failed to produce a moving boundary under similar conditions.

Electron microscopy.—Drops of the purified plant extracts were placed on parlodion film supports that had been prepared 4 days previously. The films were dried and shadowed with chromium (12) and examined under an RCA electron microscope, type EMU.

Ultraviolet absorption.—Ultraviolet absorption spectra were determined in a Beckman DU spectrophotometer in the region between 235 and 300 mμ at 5-mμ intervals.

Experimental results.—Pellets from the first 2 cycles of differential centrifugation following ammonium sulfate precipitation were brownish, but the color gradually diminished as purification proceeded further. The virus pellet at the fifth cycle was homogeneous and only faintly colored. An additional cycle did not appreciably reduce the color, and no further attempt was made to remove contaminants.

A purified virus suspension concentrated 150-fold had a protein concentration of 7.14 mg/ml. It was opalescent and colorless and exhibited intense streaming birefringence when examined between crossed Polaroid lenses. Preparations containing approximately 10 mg of protein/ml exhibited spontaneous birefringence when examined in the same manner. This anisotropic property of the virus observed early in the investigation was employed as a guide in the washing of

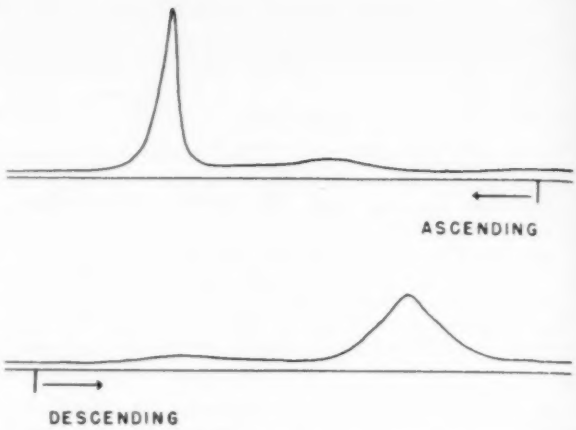

Fig. 1. Electrophoretic patterns of a purified preparation of hydrangea ringspot virus in 0.05 M phosphate buffer, pH 7.0, containing 7.14 mg of protein/ml. Electrophoresis was carried out for 180 minutes at potential gradient of 5.3 volts/cm.

sediments collected from clarification in the later phases of the purification procedure. When the control extract from noninfected plants was subjected to a similar purification procedure, a barely visible pellet that showed none of the properties possessed by the virus pellet was produced.

Electrophoresis revealed 2 distinct components in the purified virus preparation (Fig. 1). The electro-

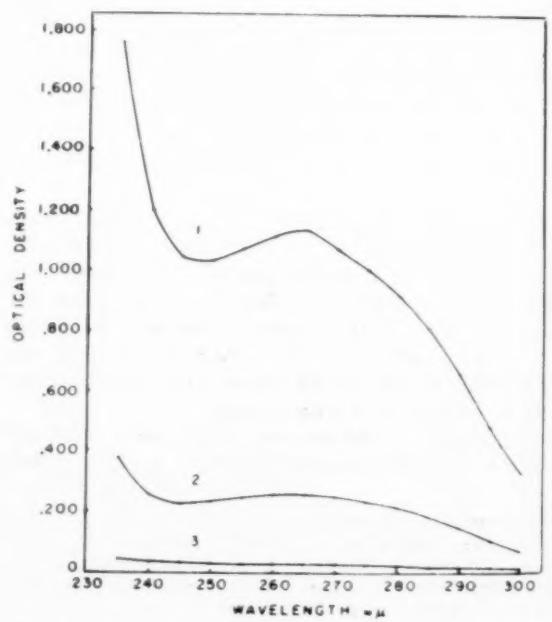

Fig. 2. Ultraviolet absorption spectra of a 1:20 dilution of a virus preparation and of different fractions of such a preparation. Curve 1) Purified virus preparation (before electrophoresis) containing 0.36 mg of protein/ml. Curve 2) Electrophoretic fast-moving ascending fraction containing 0.10 mg of protein/ml. Curve 3) Electrophoretic slow-moving descending fraction containing 0.01 mg of protein/ml.

TABLE 1.—*Infectivity, optical density, and protein content of a purified preparation of hydrangea ringspot virus and the electrophoretic mobility, infectivity, optical density, and protein content of 2 electrophoretic components*

Sample	Electrophoretic mobility, $\times 10^{-5}$ cm^2/volt/second at pH 7.0	Average number of lesions per leaf [a] at 10^{-3} dilution	Optical density at 265 mμ (1:20 dilution)	Protein, mg/ml (1:20 dilution)
Purified preparation before electrophoresis		34.6	1.132	0.36
Fast-moving ascending fraction	−3.9	9.4	0.258	0.10
Slow-moving ascending fraction	−2.2			
Fast-moving descending fraction	−3.8			
Slow-moving descending fraction	−1.7	0	0.025	0.01

[a] Ten leaves of Gomphrena used for assay of each sample.

phoretic mobilities of the 2 components in both the ascending and the descending moving boundaries were calculated from enlarged tracings of the photographs taken after 3 and 4 hours. The averages, together with data on the infectivity, optical density, and protein concentration of the fast-moving ascending fraction, of the slow-moving descending fraction, and of the sample before electrophoresis are presented in Table 1. The fast-moving ascending fraction exhibited strong anisotropy of flow, which was lacking in the slow-moving descending fraction.

The ultraviolet absorption spectrum was determined for the 2 electrophoretic fractions and for the sample before electrophoresis. The fast-moving ascending fraction and the sample before electrophoresis yielded absorption spectra characteristic of nucleoprotein, with maxima in the region of 260–265 mμ, whereas the slow-moving descending fraction did not produce such a maximum. The absorption spectrum of the purified control preparation was similar to that of the slow-moving descending fraction shown in Figure 2.

Electron micrographs showed that the purified virus preparations and the fast-moving ascending fractions contained rods and filaments, whereas the slow-moving descending fractions and the noninfected-control preparations were free of such particles. Spherical particles occasionally were found with the filaments as well as in the slow-moving descending fraction and in the noninfected control preparations. The virus particles were 16.0–16.7 mμ in width, with an average width of 16.3 mμ. The length of the particles ranged from 44 mμ to various greater lengths. They usually were aggregated lineally into filaments; some also were in lateral aggregation, usually in pairs (Fig. 3). A purfied suspension prepared from infected Gomphrena leaves was found to be infectious and to contain rods and filaments similar to those found in preparations from infected hydrangea.

Preparations that were infectious (Table 1) contained particles in the form of rods and filaments and gave ultraviolet absorption maxima between 260 and 265 mμ. Furthermore, the infectivity titer correlated well with the optical density and with the protein concentration of the suspensions.

DISCUSSION.—The results of the present study show clearly that hydrangea ringspot virus is rod shaped. No characteristic length could be determined for this virus, since particles of many lengths were present; however

FIG. 3. Electron micrograph of hydrangea ringspot virus—a purified preparation of an extract from infected hydrangea leaves, showing virus filaments with and without segments. × 90,000.

there was a tendency of the particles to segment into 44-mμ lengths or into multiples of this dimension. A 44-mμ segment is indicated by the arrow in Figure 3. Segmented filaments longer than 900 mμ and filaments longer than 800 mμ with no apparent segmentation were present. Apparently, the characteristic particle of this virus is extremely long or there was an end-to-end aggregation of shorter particles. An end-to-end aggregation of rods of potato virus X was suggested by Takahashi and Rawlins (11) and of rods of tobacco mosaic virus by Black et al (1).

The width of hydrangea ringspot virus (16.0–16.7 mμ) falls near that of tobacco mosaic virus (15 mμ) (7) and that of 2 strains of potato virus X (15.7 and 16.1 mμ) (11).

Inoculation of the 2 electrophoretic components to Gomphrena showed that the fast-moving component is the virus, with an electrophoretic mobility of -3.9×10^{-5}cm^2/volt/second in 0.05 M phosphate buffer at pH 7.0. In comparison with tobacco mosaic virus, which has an electrophoretic mobility of -9.7×10^{-5}cm^2/volt/second in 0.05 M phosphate buffer at pH 7.0 (4) and one of -8.8×10^{-5}cm^2/volt/second in 0.05 M phosphate buffer at pH 7.1 (8), the hydrangea ringspot virus is an electrophoretically slow-moving virus.

The presence of a noninfective electrophoretic component and the occurrence of occasional particles other than rods in the electron micrographs indicate that some contaminants were still present in the purified preparations. The tendency of hydrangea ringspot virus particles to form fibrous masses in which contaminants could be trapped probably accounts for the difficulty encountered in purification.

Several characteristics of this virus suggest relationship to the potato virus X group. The particles of both viruses are flexible and of the same width, and both produce the same type of lesion on *G. globosa*. Also, the thermal inactivation points for hydrangea ringspot (3) and potato virus X (6, p. 43) are similar. However, the host range, as determined by Brierley and Lorentz (3) indicates that hydrangea ringspot virus is not one of the presently described members of the potato virus X group. There is no substantial evidence presently available that would give any definite indication of a relationship to this group.

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PHYSIOLOGIC RACES OF THE RED CLOVER LEAF RUST FUNGUS[1]

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SUMMARY

Five physiologic races of *Uromyces trifolii* var. *fallens* were distinguished in collections from 14 locations in the northern United States. This constitutes the first record of physiologic specialization within *U. trifolii* var. *fallens*. Individual red clover plants were used as differentials to separate races after it was determined that commercial varieties were unsuitable for this purpose. The varieties Common Oregon and Purdue were completely susceptible to the 4 races tested on them, whereas Altaswede, Pennscott, and several other varieties were heterogeneous for resistance and susceptibility. For convenience, rust reactions were determined on inoculated detached leaflets. There was an excellent correlation between the reactions of detached leaflets and the reactions of whole plants. Inoculated leaves on potted plants were wetted by dew that formed on them after the pots had been placed in shallow water that was kept warmer than the ambient air of the enclosing container.

The red clover rust fungus, *Uromyces trifolii* (Hedw. f.) Lév. var. *fallens* (Desm.) Arth., is distinguished from other varieties of *U. trifolii* by suscept range within the genus *Trifolium* (1, 6, 8). Mains (8) suggested that there might be physiologic specialization within *U. trifolii* var. *fallens*, but there is no direct evidence for the occurrence of such specialization. Interest in this question has been renewed by Latter's (7) demonstration that there are 2 principal races of the subterranean clover rust fungus, *U. trifolii* (Hedw. f.) Lév. [var.?] *subterranei*,[2] in Australia and that subterranean clover (*T. subterraneum* L.) varieties resistant to the widespread Race A are susceptible to Race B. The present paper reports the first demonstration of the existence of physiologic races within *U. trifolii* var. *fallens*.

MATERIALS AND METHODS.—Red clover (*Trifolium pratense* L.) plants were grown singly in 3.5-in. pots in a greenhouse from which rust was excluded. Nine lines derived from single urediospores, 6 lines derived from single uredial pustules, and 5 nonpurified "bulk" lines of *U. trifolii* var. *fallens* were isolated from rusted red clover leaves collected from 14 locations in the northern United States. The lines were cultured in the uredial stage on detached red clover leaflets. Each line was transferred every 2 or 3 weeks to leaflets freshly detached from 5 randomly selected plants.

For detached-leaflet inoculations, unblemished leaflets that were nearly mature were excised from leaves in the laboratory and rubbed gently between the fingers. Urediospores were brushed on lower surfaces with a dry camel's-hair brush. Numerous minute water droplets were sprayed on with a hand atomizer. Inoculated leaflets were floated on a solution of 2 per cent sucrose in sterilized distilled water in Petri dishes. Five leaflets, each from a different plant, were placed in each dish. Cultures were kept in incubators at 20°–22° C under continuous fluorescent light of 130–185 foot-candles. These conditions permitted good infection, and the leaflets remained green and mold free for approximately 3 weeks.

Fourteen to 17 days after inoculation of the detached leaflets, infection types were determined according to the following scale: 0 = immune—no uredia; 0; = hypersensitive flecks but no uredia; 1 = very resistant—uredia minute, about 0.1–0.2 mm in diameter; 2 = moderately resistant—uredia small to medium, about 0.3 mm in diameter; 3 = moderately susceptible—uredia medium in size, about 0.4–0.5 mm in diameter; 4 = very susceptible—uredia large, about 0.6 mm in diameter or larger. Density of necrotic flecks or pustules was nearly always 80–120 per cm^2 and therefore was not a measure of susceptibility.

Whole plants to be inoculated were trimmed to 5 or 6 healthy mature basal leaves, and urediospores were brushed on. Inoculated leaves were wetted by inducing dew to form upon them. To accomplish this, an inoculated plant was placed in a cylindric battery jar containing 0.5 in. of water. The open top end of the jar was covered with aluminum foil fastened with a rubber band, and the bottom 0.5 in. of the jar was immersed in a water bath at 33° C. The top 5 in. protruded through a hole in the cover of the bath into cooler air at approximately 26° C. Dew formed within 12 hours. During warm weather, the apparatus was used in a 6° cold room and the water bath kept at 30° C. Under these conditions, dew formed in about 2 hours. After dew had formed, the jars were placed in the laboratory. Twelve hours later covers were removed for a minute, replaced for an additional 18 hours, and then removed permanently. Plants were kept on a laboratory table until infection data were taken 15–21 days after inoculation. They received only the daylight entering a laboratory window. Temperature in the room varied from 24° to 30.5° C.

RESULTS.—*Comparison of detached-leaflet reaction with whole-plant reaction.*—The detached-leaflet technique makes it possible to make rapid simultaneous tests of the reaction of genetically identical material,

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Based on an M. S. thesis. Appreciation is expressed to D. A. Roberts, under whose direction this work was completed, and to W. K. Kennedy, for helpful suggestions during the research and preparation of the paper.

[2] Called *U. trifolii subterranei* by Latter (7). Authorities for trinomial nomenclature by Latter or for the varietal designation *subterranei* were not found in the literature.

such as a single plant or clone, to many different isolates. Such tests can be made under controlled conditions and with a minimum danger of contamination (11). These advantages recommended the use of this technique in the present problem, provided that detachment did not alter inherent susceptibility or resistance of the leaflets.

To test the reliability of the detached-leaflet technique for the determination of the reaction of red clover to the rust fungus, some leaflets were detached from each of 47 plants selected at random from several varieties and the remaining leaflets were inoculated with randomly selected lines of *U. trifolii* var. *fallens*. At the same time, the detached leaflets from these plants were inoculated with the same lines of the fungus.

The results showed conclusively that suceptibility or immunity of red clover plants to rust can be determined by the reaction of their detached leaflets. Invariably, when pustules developed on a plant they also developed on the leaflets detached from that plant. When no pustules formed on a plant, none formed on leaflets that had been detached from it. Forty-two plants became infected; of these, 24 bore pustules equal in size and number to those upon their detached leaflets. Pustules on the other 18 plants were somewhat smaller than those on their detached leaflets. Pustules usually enlarged more slowly on attached than on detached leaflets. Probably, pustule growth was favored more by the continuous light, supplemental sugar, and moderate temperatures provided the detached leaflets than by the poor light and rather high temperature attending incubation of whole plants. The fact that detached-leaflet and whole-plant infection (or lack of infection) correlated perfectly in spite of the different environmental conditions emphasizes the validity of the detached-leaflet method for assessing whole-plant reaction.

Comparison of varietal reaction with individual plant reaction.—Red clover is cross-pollinated, and commercial varieties are heterogeneous aggregations of many genotypes (9). It was anticipated, therefore, that physiologic races of the red clover rust fungus might not be distinguishable by their reactions on commercial varieties but that they could be distinguished by their reactions on individual plants. This assumption was tested by determining the reaction of individual plants of 4 varieties to 4 pure lines of the rust fungus. Thirty plants were randomly selected from each of the varieties Pennscott, Altaswede, Purdue, and Common Oregon. The reaction of each plant to all 4 lines of the rust fungus was determined by the detached-leaflet technique. Four leaflets from each plant were inoculated at different times with each line of the fungus.

It was not possible to differentiate among the 4 lines by the reactions of these varieties to them. All 30 plants of Common Oregon were highly susceptible to all 4 lines. Twenty-eight plants of Purdue were moderately to highly susceptible, 1 was resistant, and 1 was immune. From 19 to 23 plants of Pennscott and Altaswede were susceptible to any given line; the remainder were either resistant or immune.

TABLE 1.—*Infection types induced by 4 single-urediospore lines of Uromyces trifolii var. fallens on detached leaflets of 30 plants of Altaswede red clover and of 30 plants of Pennscott red clover, with plants of each variety grouped according to their reactions to the 4 lines*

Variety	Group	Infection type[a] following inoculation with indicated line[b]				No. of plants in group
		NY	PA	IND	ORE	
Altaswede	1	3–4	3–4	3–4	3–4	16
	2	0	0	0	0	4
	3	0	0	0	4	3
	4	4	4	4	2	2
	5	3	4	0	3	1
	6	3	2	1	3	1
	7	1	3	3	3	1
	8	3	3	0	0	1
	9	2	0	0	3	1
						30
Pennscott	1	3–4	3–4	3–4	3–4	17
	2	0	0	0	0	6
	10	4	4	4	0	1
	11	4	4	3	0	1
	12	0	3	0	0	1
	13	0	0	1	3	1
	14	3	1	0	4	1
	15	3	4	2	3	1
	16	3	3	1	3	1
						30

[a] Infection types from 0 (immune) to 4 (highly susceptible).

[b] NY isolated from collections from Duanesburg, New York; PA from State College, Pennsylvania; IND from Lafayette, Indiana; and ORE from Corvallis, Oregon. Later these were assigned, respectively, to Races 4, 3(?), 2, and 5.

When the effects of the 4 lines upon individual plants of Pennscott or Altaswede were compared, however, physiologic differences among the lines became apparent (Table 1). Both the Pennscott and Altaswede varieties include numerous genotypes for resistance and susceptibility. Less extensive tests showed a similar heterogeneity in other varieties, including Common Italian, Aberystwyth S-151, Svalof, Manhardy, 151 Uruguay, Dollard, Midland, Common Mammoth, Alaska, Common Danish, and a selection of Wisconsin Anthracnose Resistant. Data in Table 1 indicate that physiologic races can be distinguished by the use of individual plant differentials.

Selection of differential plants.—One hundred sixty-nine red clover plants taken from 34 varieties were screened by detached-leaflet inoculation for their suitability as differential plants. Four to 10 plants of a variety were tested in random combinations with 2–9 lines of the fungus per plant in a total of 719 replicated combinations. One hundred forty-one plants were susceptible and 14 were resistant to all the lines with which they had been inoculated. The remaining 14 plants were susceptible to some lines and resistant to others. Leaflets from each of these plants were inoculated separately with each of 17 lines, and plants hav-

TABLE 2.—*Infection types produced by 5 physiologic races of Uromyces trifolii var. fallens on detached leaflets of 6 differential plants of Trifolium pratense*

Physiologic race	Infection type on indicated differential plant[a]					
	A	B	C	D	E	F
1	4	4	4	0;	0	0;
2	4	4	0;	0;	0	0;
3	4	4	0	4	0	0;
4	4	0;	0;	0;[b]	0	0;
5	0	4	4	0;	3	4

[a] Each differential plant was an individual selected from 1 of 6 varieties. Infection types ranged from 0 (immune) to 4 (highly susceptible), with 0; indicating hypersensitive flecking.

[b] Occasionally type 1 infection.

ing intermediate or variable reactions were discarded. Six plants that were consistently immune or highly susceptible to all lines tested were chosen to be the differentials; these differentials, designated A, B, C, D, E, and F, were individual plants selected from Svalof, Pennscott (F.C. 24282),[3] Wisconsin Anthracnose Resistant (1952 crop, not released), Common Mammoth (F.C. 24295),[3] Common Italian (F.C. 23224),[3] and 151 Uruguay (F.C. 23453),[3] respectively.

Demonstration of physiologic races.—Leaflets detached from each of the 6 differential plants were inoculated separately with each of the 15 pure lines and with each of the 5 "bulk" lines of *U. trifolii* var. *fallens*. Every possible combination of the 6 plants and 20 lines was made in duplicate 3 or more times.

The lines fell into 5 distinct groups; these groups were considered to be physiologic races and were designated Races 1, 2, 3, 4, and 5 of *U. trifolii* var. *fallens*. The infection type induced by a given race on a given differential was constant, with the exception that Race 4 sometimes gave type 1 instead of type 0; on differential D. Infection types induced by the 5 races on detached leaflets of the 6 differentials are shown in Table 2. Typical reactions of detached leaflets of differentials A, B, C, and D to the 5 races are illustrated in Figure 1.

The geographic origin of the 14 collections of *U. trifolii* var. *fallens* and the races isolated from them are shown in Table 3. It was not ascertained whether or not more than 1 race occurred in collections from which only 1 monospore line had been isolated. Disposition of the pure line from Pennsylvania was not clear. The infection type on B and D varied from 0; to 3 for no discernible reason. The line might be a biotype of either Race 3 or Race 4, or it might belong in a race different from any of the 5 designated. All other lines tested were racially pure.

Inoculations of differentials as entire plants confirmed the assumption that they would react to different physiologic races in the same manner as did their detached leaflets. Five or 6 leaves on one side of an aluminum foil divider were brushed with spores of a race presumed to be pathogenic, and 5 or 6 leaves on the other side were brushed with spores of a race presumed to be nonpathogenic according to detached-leaflet reactions that had been determined previously. Leaflets detached from the plant were inoculated with the same 2 races. Plants were wetted by the dew-induction technique and were kept in lighted cabinets at 22°C. In every instance, it was found that infection occurred after 15 days on leaves inoculated with the presumably pathogenic race and did not occur on leaves inoculated with the presumably nonpathogenic race. Pustule size on the whole plant differentials usually was slightly smaller than on detached leaflets, so that infection type was ½ to 1 grade lower when graded by detached-leaflet definitions than when graded on whole plants.

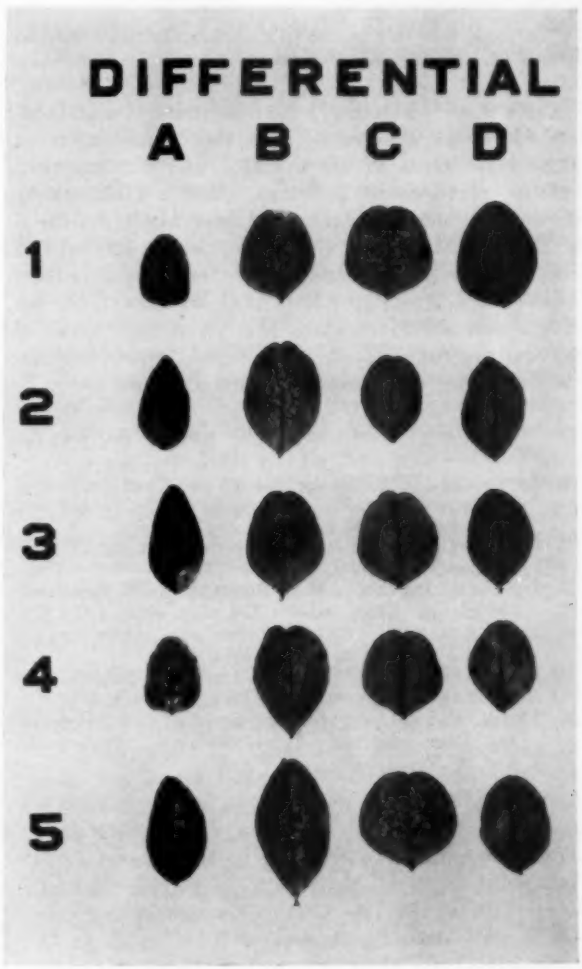

FIG. 1. Typical reactions of detached leaflets of differential red clover plants A, B, C, and D inoculated with Races 1, 2, 3, 4, and 5 of *Uromyces trifolii* var. *fallens*. Each differential was an individual plant selected from 1 of 6 varieties. Large spots on margins of leaflets of differentials B, C, and D were made with India ink for purposes of identification. (Photograph by H. H. Lyon, Jr.)

[3] Identification number of the U. S. Department of Agriculture, Field Crops Research Services.

TABLE 3.—*Races of Uromyces trifolii var. fallens present in 20 isolates derived from 14 collections of the red clover rust fungus*

Source of collection	Nature of isolates[a]	Designation of races present
Kingston, R. I.	S	2
Duanesburg, N. Y.	S	4
Malone, N. Y.	S	4
Ithaca, N. Y.	S	4
Ithaca, N. Y.	B	2
Rochester, N. Y.	S, P, P	2, 3, 4
State College, Pa.	S	?[b]
Linworth, Ohio	P, P	1, 2
Linworth, Ohio	P, P	1, 2
Washington Court House, Ohio	B	2
Lexington, Ky.	B	2
Lafayette, Ind.	S, B	2
Madison, Wis.	S	4
Corvallis, Ore.	S, B	5

[a] S, a single-urediospore line; P, a single-pustule line; B, a nonpurified "bulk" line.

[b] Disposition of this isolate is uncertain. It may belong in Race 3 or 4, or in a different race.

DISCUSSION.—Latter (7) used varieties of subterranean clover as differentials for the identification of physiologic races of *U. trifolii* [var.?] *subterranei*, because reactions of individuals within each variety were nearly identical, except for those within the variety Bacchus Marsh. In the present study, several red clover varieties were found to be heterogeneous in their reaction to different pure lines of *U. trifolii* var. *fallens*. It was concluded, therefore, that physiologic races of the leaf rust fungus could not be distinguished on commercial red clover varieties. Subterranean clover is self-pollinated, and a variety or strain is uniform, comprising essentially a single genotype (4, 10). Red clover is cross-pollinated, is nearly completely self-sterile, and is necessarily genetically heterogeneous (9). Any plant may differ from any other regarding its factors for susceptibility and resistance; therefore, to provide uniform genotypes against which to test lines of a pathogen, individual plants, rather than varieties, had to be used as differentials.

With the modern practice of improving forage crops through genetics and plant breeding, varieties may be derived from a few plants or strains of proved superiority; in such cases, susceptibility of a variety as a whole would be determined by the susceptibility of a few individuals. The variety Purdue was derived from 3 closely bred selections (2). As this study showed, Purdue is highly susceptible to the races of *U. trifolii* var. *fallens* occurring in its area of adaptation. Evidently, none of the 3 parental selections was rust resistant. In contrast to this, it is noteworthy that Pennscott, the result of natural mass selection (5), and Altaswede, the result of natural and artificial mass selection (3), have a fair proportion of plants with resistance or immunity to several races. The detached-leaflet technique offers a rapid method for characterizing red clover parental material for its leaf rust resistance, and it facilitates the procedures used in the incorporation of resistance to known races of the rust fungus into new varieties.

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FACTORS AFFECTING DEVELOPMENT OF STALK ROT OF CORN CAUSED BY DIPLODIA ZEAE AND GIBBERELLA ZEAE[1]

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SUMMARY

Stalk rot of corn caused by *Diplodia zeae* (Schw.) Lev. and *Gibberella zeae* (Schw.) Petch. reduced yielding ability of corn from 3 to 18 per cent over a 3-year period. Yields varied with the variety of corn, the pathogen, the number of infections, the part of plant infected, and the year. Two isolates each of *D. zeae* and *G. zeae* caused no apparent antagonism or synergism when introduced together at the same point in the host.

Stalk rot was greater in smutted plants and in plants with leaves removed prior to inoculation than in the controls. There was more rot in corn grown in the greenhouse at 85° than at 65°F. Less rot developed in plants growing in flooded plots and in plants with ears removed than in the controls. The boring of holes in the stalk and the cutting of roots to simulate the effect of corn borers and rootworms had no apparent effect on the development of stalk rot. Previous infection of the plant with a stalk-rotting organism did not influence the amount of rot from later infections by the same organism. Corn was most susceptible to stalk rot when inoculated just before the time of pollen production and thereafter.

Stalk rot of corn, caused by a number of different pathogens, is of major importance in the production of corn in Minnesota. Various workers (4, 5, 6, 12, 13, 17) in the corn belt have estimated reductions in potential yield of 2 to 20 per cent, depending on the severity of infection. Rotted stalks frequently break over as the plants mature, and the yield of grain is usually reduced materially. Broken stalks also make mechanical harvesting difficult, but such losses may be avoided in part by hand picking or by permitting animals to graze the harvested fields. Stalk rot of corn has been known for more than 40 years (1, 7, 8), but little is known of the effect on yield and of the effect of factors influencing disease development. The present study was undertaken to determine to what extent stalk rot reduces the yielding ability of corn and to determine some of the factors that affect the development of stalk rot in corn.

FUNGUS CULTURES, INOCULATIONS, AND DISEASE RATING.—Cultures derived from single spores of 2 isolates of *Diplodia zeae* (Schw.) Lev., D1 and D2, and of *Gibberella zeae* (Schw.) Petch., G1 and G2, were used. Inoculum was prepared by the toothpick method of Young (19). After the fungus had grown on the toothpicks for about 2 weeks, the infested toothpicks were inserted into stalks of corn. Disease ratings were made by cutting the stalk lengthwise. Pathogenicity ratings were determined as follows: 1) infections involving up to 25 per cent of an internode; 2) 26-50 per cent; 3) 51-75 per cent; and 4) 76-100 per cent. When infection extended beyond the internode inoculated, the rating given was equal to the sum of the ratings of the internodes infected.

YIELD TESTS.—Minhybrids 504 and 607 were inoculated with *D. zeae* and *G. zeae* in the second internode above the ground, in the internode below the shank or ear shoot, in the shank, and in various combinations of these 3 loci. Plants were inoculated with a single species about 10 days after pollination. The experiment was set up in a randomized block, replicated 3 times with 15 3-plant hills per treatment. The controls were not inoculated. All ears were harvested and the yield was determined in bushels per acre.

The inoculated corn yielded less than the noninoculated controls (Table 1). The reduction in potential yield due to stalk rot varied with the hybrid, the location of the infection, and the number of infections. In general, infection had a greater effect on the yield of Minhybrid 504 than on that of Minhybrid 607 when inoculation was made at a single point; results were more variable with *G. zeae* than with *D. zeae*. Percentage reduction due to *D. zeae* ranged from 10.4 to 11.6 and from 7.5 to 9.9 for Minhybrid 504 and Minhybrid 607, respectively; that due to *G. zeae* ranged from 5.1 to 9.1 and from 3.2 to 8.4 for the respective hybrids. Single inoculations regardless of location had about equal effect on yield. Inoculations at more than 1 point usually resulted in a slightly greater influence on yield than did inoculations at 1 point, but in some cases yield was greater in plants with 2 points of inoculation than with a single point.

There was a variation in yield also from year to year. In 1951, reduction in potential yield due to stalk rot was less than in either 1950 or 1952, particularly with *G. zeae*. In 1951 the reduction ranged from 0 to 5.5 per cent for Minhybrid 607 when inoculated with *G. zeae*, whereas in 1950 the range was from 5.4 to 21.5 per cent.

The many uncontrolled factors that influence the development of stalk rot in corn may account for the pronounced seasonal effect.

FACTORS AFFECTING THE DEVELOPMENT OF STALK ROT.

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TABLE 1.—*Three-year averages of yield of 2 corn hybrids when the stalks were inoculated with 2 fungi in the second internode above the ground, in the shank internode, in the shank, and in various combinations of these 3 loci*

Point of inoculation	Yield in bushels[a] per acre for indicated hybrid when inoculated with indicated fungus				Mean both hybrids and fungi
	Diplodia zeae		*Gibberella zeae*		
	Minhybrid 504	Minhybrid 607	Minhybrid 504	Minhybrid 607	
Second internode	50.2	53.5	51.3	53.5	52.1
Shank internode	50.6	53.2	53.6	55.9	53.3
Shank	49.9	52.1	52.2	52.9	51.5
Second and shank internode	49.4	53.5	52.0	53.2	52.0
Second internode and shank	46.6	54.2	47.7	53.9	50.6
Shank internode and shank	46.0	50.8	47.9	51.7	49.1
Second and shank internode and shank	49.1	50.8	49.7	49.5	49.8
Noninoculated control	56.5	57.8	56.5	57.8	57.1

[a] Means for 3 years, with 3 replications of each treatment of 15 3-plant hills. LSD at 5 per cent level is 2.5 bushels.

—To determine the effect of the association of different isolates of *D. zeae* and of *G. zeae* on the development of stalk rot, Minhybrids 504 and 607 were inoculated with different cultures of the 2 organisms, singly and in combinations. Inoculations were made in the same internode, either at the same point or about 2 in. apart.

The results were based on 10-hill rows of 3 plants per hill; the tests were replicated twice per treatment in each of 3 consecutive years. Infection ratings were made 3 weeks after inoculation. With 2 pathogens introduced at the same locus, the amount of rot was approximately the same as when only the more virulent pathogen of the combination was introduced (Table 2.) Infection ratings were greater when inoculation points were separated than when they were not; this probably was due to the coalescing of the 2 infected areas and not due to one pathogen stimulating the other.

TABLE 2.—*Stalk rot ratings in 2 corn hybrids when each plant was inoculated with 2 isolates of Diplodia zeae and with 2 isolates of Gibberella zeae, either singly or with paired isolates of the 2 species*

Organism	Isolate	No. of inoculation points[a]	Ratings[b] in indicated hybrids	
			Minhybrid 504	Minhybrid 607
D. zeae	D1	1	3.0	3.0
	D2	1	3.2	3.4
G. zeae	G1	1	2.5	2.2
	G2	1	1.9	2.4
Both	D1 + G1	1	2.8	2.9
		2	3.3	3.4
	D1 + G2	1	3.0	3.2
		2	3.4	3.6
	D2 + G1	1	3.1	3.6
		2	3.6	3.9
	D2 + G2	1	3.0	3.4
		2	3.5	3.7

[a] Where 2 inoculation points are indicated, each isolate was introduced at different points 2 in. apart.

[b] Means for 3 years, with 2 replications of each treatment each year. Ratings based on percentage of internode infected as follows: 1 = 0-25; 2 = 26-50; 3 = 51-75; 4 = 76-100. LSD at 5 per cent level is 0.5.

The effect of a saturation of soil with water on the development of stalk rot was determined. Inoculations were made in the second and fifth aboveground internodes of 2 corn hybrids. Half of the tests were made in a plot flooded with 3–4 in. of water about 2 weeks before inoculation. Water was added to the plots when puddles were almost gone. Water was standing on the ground until rot ratings were made. Soil moisture in the nonflooded plots was adequate for good growth of the corn plant. The test comprised 2 replications of each treatment consisting of 10 plants inoculated with each of the 2 organisms. Disease ratings were made 3 weeks after inoculation. The greatest amount of rot was in the nonflooded plots (Fig. 1).

Over a period of years at St. Paul, Minnesota, stalk rot appeared to be least severe in comparatively cool summers. Likewise, Melhus and Durrell (15) reported a high incidence of stalk rot in 1907 and 1921, years of high summer temperatures, and a low incidence in 1915, a year of low summer temperatures; heavy rainfall occurred in all 3 years. There are, however, no experimental data on the effect of temperature on stalk rot. Therefore, tests were conducted in the greenhouse at 65° and 85°F to determine the effect of temperature on the development of this disease.

The test comprised 56 pots, each containing 3 plants of Minhybrid 607. The corn was grown at 85° until the time of pollen production; at this time, the plants in half the pots were inoculated with *D. zeae* and those in the remaining pots with *G. zeae*. Half of the pots of each group were transferred to 65° and the remainder kept at 85°. On the seventh day after inoculation and every other day thereafter until 33 days after inoculation, 3 plants inoculated with each organism from each temperature were examined to determine the extent of the rotted area above and below the point of inoculation. Extent of rot was determined both by measuring the amount of rot and by culturing serial sections of the stalk. The data for both pathogens fall on similar curves for the 2 temperatures, but the amount of rot was greater at the higher temperature

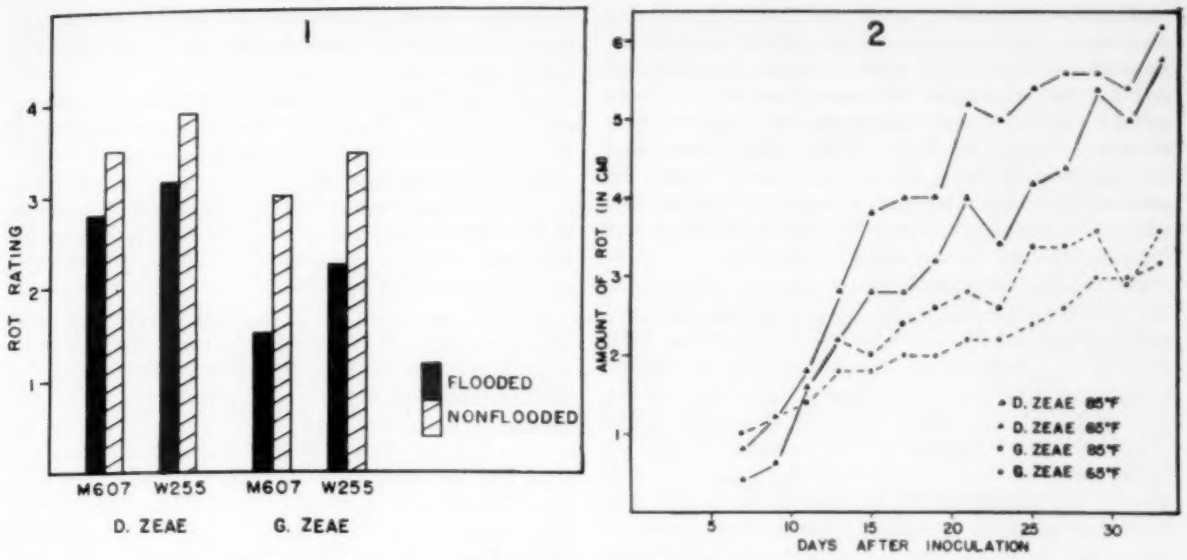

FIG. 1-2. Comparison of stalk rot rating in corn hybrids Minhybrid 607 and Wisc. 255 inoculated with *Diplodia zeae* and *Gibberella zeae* when grown in flooded and nonflooded plots. Ratings based on percentage of internode infected as follows: 1 = 0–25; 2 = 26–50; 3 = 51–75; 4 = 76–100.—Fig. 2. Amount of stalk rot above and below the point of inoculation with *Diplodia zeae* and *Gibberella zeae* in Minhybrid 607 growing in the greenhouse at 65° and 85° F.

(Fig. 2).

Although numerous investigators (6, 9, 10, 12, 14, 16) have reported stalk rot primarily as a disease of corn approaching maturity, many observations indicate that infection may be present much earlier. To determine at what stage in the development of corn the plant is most susceptible, inoculations of Minhybrids 504 and 607 and Wisc. 255 were made with *D. zeae* and with *G. zeae* at weekly intervals from June 16 to September 8, 1952. Ten stalks inoculated with each organism were examined at intervals of 2 weeks until September 23.

Results with the 3 hybrids were similar, hence only those for Minhybrid 504 are reported (Fig. 3). Ratings for inoculations made previous to July 7 are not represented, because the infested toothpick, when inserted into the rapidly developing plant, frequently did not come in contact with the stem.

The results indicate that corn is susceptible to stalk rot over a considerable period of time, with the period of greatest susceptibility beginning several weeks before pollen production and extending into the hard dough and dent stages. In 1952, the peak of pollen production was about August 4; however, this varied with the varieties. It is noteworthy that infection from earlier inoculations did not become so extensive as did infection from later inoculations, even though there was a longer period for rot development. This was true for all 3 varieties inoculated with both *D. zeae* and *G. zeae*.

It is not uncommon to find more than 1 disease present on the same plant, with 1 disease influencing the development of the other (9, 11, 17). Inoculations with *D. zeae* and *G. zeae* were made in stalks of Minhybrid 607 and Wisc. 255 with a large smut gall (caused by *Ustilago maydis* (DC.) Cda.) on the upper part of the stalk and in nonsmutted adjacent stalks. Approximately 25 such paired inoculations were made in each of 2 varieties with each fungus. Internal stalk rot ratings were made 3 weeks later. The smutted

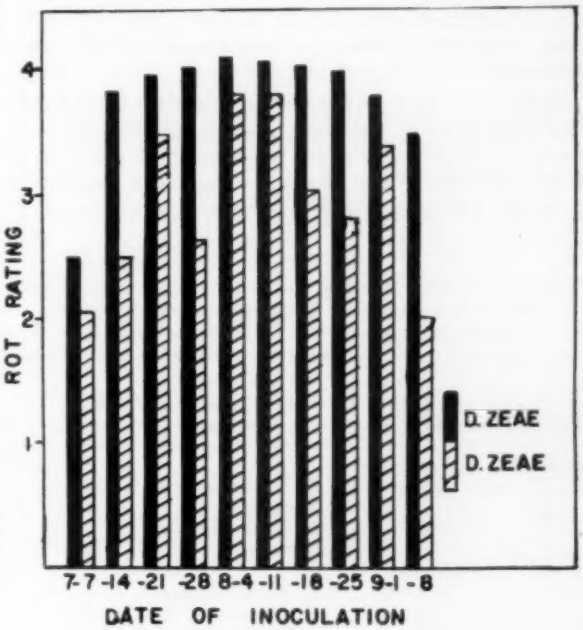

FIG. 3. Stalk rot rating on September 23, 1952, in Minhybrid 504 when inoculated with *Diplodia zeae* and *Gibberella zeae* at weekly intervals from July 7, 1952, to September 8, 1952. Ratings based on percentage of internode infected as follows: 1 = 0–25; 2 = 26–50; 3 = 51–75; 4 = 76–100.

stalks were consistently more susceptible to stalk rot than were the nonsmutted ones. Mean ratings for smutted and nonsmutted stalks, respectively, were 3.7 and 3.2 for Minhybrid 607 inoculated with *D. zeae* and 3.2 and 2.5 for the same variety inoculated with *G. zeae*. Likewise, for Wisc. 255 the ratings for smutted and nonsmutted stalks, respectively, were 4.0 and 3.6 when inoculations were with *D. zeae* and 3.4 and 2.9 when inoculations were with *G. zeae*. A difference of 0.3 was required for statistical significance.

To simulate the effect of leaf blight, Minhybrids 504, 607, and 706, and Wisc. 240 were defoliated to different degrees. Leaves were broken halfway back from the tip of the blade or removed 2 weeks prior to inoculation, and similar treatments were applied the day of inoculation. There were 2 replicates of 15 plants for each treatment. The results (Table 3) varied with the variety and with the fungus, but in general the amount of rot was greater in plants with the leaves removed or broken 2 weeks prior to inoculation than in plants not injured prior to inoculation. There was little or no difference in the amount of rot in the controls and in the plants multilated the day of inoculation. A difference in rating of 0.3 was required for statistical significance.

In another test, all ears were removed from stalks of Minhybrid 607 and Wisc. 255 2 weeks prior to inoculation, with *D. zeae* or with *G. zeae*, in the second and fifth internodes above the ground. There were 3 replications of 10 plants in the test. Although differences were not statistically significant, consistently there was less rot in the stalks with no ears than in the controls. Holbert et al reported less rot when ears had been removed in stalks inoculated with Diplodia.

In the field, all infections of a single plant undoubtedly do not occur at the same time. It was thought that early infection might affect the amount of rot from a later infection. When plants that had been inoculated 2 weeks previously were inoculated with the same organisms, however, the amount of rot was not greater than in controls inoculated only at the time of the second inoculation.

Stalk rot frequently follows stalk damage caused by the European corn borer, *Pyrausta nubilalis* Hbn. (2, 3, 18). *Diabrotica longicornis* Say. and *D. undecimpunctata howardii* Barb., the northern and southern corn rootworms, respectively, frequently destroy much of the root system. The mechanical damage caused by the borers and the rootworms possibly may affect the development of rot materially.

To test the effect of mechanical damage by corn borers on the development of stalk rot, a test was made with Minhybrids 504 and 607 and Wisc. 255. Following surface treatment of the stalk with 70 per cent ethyl alcohol, a horizontal hole approximately 6 mm in diameter was cut with a sterilized cork borer into the stalk but not completely through the stalk. The hole was covered with grafting tape to prevent contamination. Inoculations were made in internodes above a single hole, below a single hole, above 2 holes, and below 2 holes. Control plants had no holes bored in them. There were 2 replications of 10 plants for each treatment. There were no differences in the amount of rot in the treated and in the control plants.

When approximately 50 per cent of the roots of Minhybrid 607 and Wisc. 255 had been cut with a knife 3 weeks prior to inoculation, the amount of stalk rot was not influenced by the loss of roots because there was adequate soil moisture. Many tests made at St. Paul, however, indicate that plants inoculated during periods of insufficient soil moisture are readily killed prematurely by stalk rot.

Discussion.—Many factors apparently influence the development of stalk rot and the effect of the disease on the yield of the crop. As expected, the loss due to stalk rot varied with the corn hybrid, the pathogen, the number of infections, the location of infection, and the year.

Stalk rot is caused by several fungi, and frequently

Table 3.—*Stalk rot ratings following inoculations with Gibberella zeae and with Diplodia zeae of uninjured plants of 4 corn hybrids and of comparable plants from which leaves on 1 side of each plant had been removed or on which leaves had been broken halfway back from the tip either 2 weeks prior to inoculation or on day of inoculation.*

Pathogen	Type of injury to leaves	When injured in reference to inoculation	Stalk rot rating[a] on indicated hybrid				
			Wisc. 240	Minhybrid 504	Minhybrid 607	Minhybrid 716	Mean
D. zeae	None		3.6	3.0	3.2	3.0	3.2
	Removed	2 Weeks before	3.9	3.3	3.7	3.5	3.6
		Same day	3.5	3.0	3.4	3.2	3.2
	Broken	2 Weeks before	3.9	3.5	3.5	3.3	3.6
		Same day	3.9	2.7	3.2	3.3	3.3
G. zeae	None		2.6	2.4	2.5	2.0	2.4
	Removed	2 Weeks before	3.1	2.4	2.7	2.4	2.7
		Same day	2.6	1.8	2.4	2.1	2.2
	Broken	2 Weeks before	2.8	2.1	2.5	2.9	2.6
		Same day	2.7	2.1	2.4	2.4	2.4
		Mean	3.3	2.6	3.0	2.8	

[a] Each figure is the mean of 2 replications of 15 plants each. Ratings are based on percentage of internode rotted as follows: 1 = 0-25; 2 = 26-50; 3 = 51-75; 4 = 76-100.
LSD at 5 per cent level is 0.3.

both *D. zeae* and *G. zeae* can be isolated from the same stalk. It would seem from present tests that 1 rot does not influence the development of the other and that the influence of stalk rot in 1 part of the host does not influence the development of stalk rot in another part. Therefore, resistance of corn to several stalk rotting pathogens could be determined simultaneously without greatly increasing the amount of labor involved.

Damage, *per se*, from corn borers apparently does not directly affect the development of stalk rot; however, corn borers, and perhaps other insects, are important factors in inoculation, since the larvae provide avenues of entrance for pathogens, and they also distribute inoculum while tunneling in the stalk within internodes and from 1 internode to another. In Minnesota, rarely does one find corn infested with corn borers that does not also have stalk rot.

Corn smut has been known to predispose corn to ear rot (11); this study indicates that corn smut predisposes corn to stalk rot caused by both *D. zeae* and *G. zeae*. Corn affected by bacterial blight has been reported to be more susceptible to *D. zeae* than in corn not so affected (9). In 1949, the unusually high incidence of stalk rot in Illinois was attributed to the predisposing influence of leaf blight caused by *Helminthosporium turcicum* Pass. (17). The fact that plants with leaves removed were more susceptible than were the controls suggests that plants with leaf diseases and plants damaged by high winds or hail would be more susceptible to stalk rot than are uninjured plants.

The stage of development of the host influences its susceptibility to stalk rot. The data suggest that once the corn has reached a certain stage of maturity, the susceptibility does not change. Early inoculations resulted in smaller areas of infection than did late inoculations, even though the length of time for disease expression was greater with the former. Conceivably, the host possesses some substance that inhibits the development of the disease and from which the pathogen never fully recovers such that it may develop at the same rate as it would if introduced later.

Biological and environmental factors vary from year to year. Thus in order to estimate potential disease development and potential disease losses, tests should be made to determine the development of stalk rot under as many biological and climatic environments as possible.

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THE EFFECT OF SEED TREATMENT ON SEEDLING STAND AND FORAGE YIELD OF ALFALFA AND RED CLOVER[1]

Kirk L. Athow[2]

SUMMARY

The seedling stand from Arasan-treated and untreated seed of 64 lots of alfalfa and 61 lots of red clover were compared in field plantings. The stand of approximately 14 per cent of the alfalfa lots and 5 per cent of the red clover lots were significantly increased by seed treatment. The poor germinating lots of seed were benefited no more than were the better germinating lots.

No statistically significant differences in yield were found between treated (Arasan or Vancide 51) and untreated alfalfa and red clover seed planted in each of 4 years in plots 3 × 25 ft. at rates of seeding from 2 to 12 lb. per acre. The highest yields were usually obtained at the heavier seeding rates. Differences in yield between treated and untreated seed were no greater at the lower seeding rates, where small differences in stand would be more likely to influence the yield, than they were at higher seeding rates.

The advisability of chemical treatment of alfalfa and red clover seed still is questionable despite the information reported during the past 20 years. The literature has been reviewed adequately by Vlitos and Preston (9), Kreitlow, Garber, and Robinson (7), Kernkamp (6), and, more recently, by Tyler, Murphy, and MacDonald (8). The last-named authors pointed out that tests performed in greenhouses often resulted in benefits from seed treatment, that adequate tests had not been performed in the field, and that there were no published records of attempts to correlate yields of hay with seed treatment.

Tyler et al (8) presented field data which showed that treating seed of alfalfa, red clover, birdsfoot trefoil, orchard grass, smooth bromegrass, Sudan grass, and timothy with Arasan or with Phygon did not increase seedling stands or hay yields above stands and yields obtained from untreated seed. The studies reported here were initiated in 1952; although restricted to alfalfa and red clover, they had essentially the same purpose as did those of Tyler et al; namely, to determine the effect of chemical treatment on seedling stand and forage yield in the field.

MATERIALS AND METHODS.—The effects of seed treatment on seedling stand and forage yield were studied in separate plots because of the difficulty in obtaining accurate stand data in plots suitable for yield determinations. The seedling stands were compared from treated and untreated seed of 64 lots of alfalfa and 61 lots of red clover selected on the basis of differences in germination. Seed was treated with Arasan dust (50% bis(dimethylthiocarbomoyl) disulfide) at 8 oz. per 100 lb. of seed, since preliminary tests had shown it to be equal or superior to other materials tested. The seeds were planted 200 per 4 ft. row in a randomized split-plot design with 10 replications. Stand counts were made 4–6 weeks after planting.

Different single lots of alfalfa and red clover seed were used in each of the 4 years for the yield trials. The seed was treated with Arasan dust at the rate of 8 oz. per 100 lb. of seed or dipped in full strength Vancide 51 (27.6 % sodium dimethyldithiocarbamate and 2.4 % sodium derivative 2-mercaptobenzothiazole) for 2 minutes. The seed was planted with a Planet Jr. seeder in 5-row plots, with the rows 7 in. apart and 25 ft. long. Seeding was at the rate of 2, 4, 6, 8, and 10 lb. per acre in 1952 and 6, 8, 10, and 12 lb. per acre in 1954-1956 in a split-plot design with 4 replications. The plots were over-seeded with oats as a nurse crop. The oats were cut and removed in early June when the alfalfa and clover had become well established. The plots were harvested for forage yield at the appropriate times during the second year. Two cuttings of red clover were obtained each year. The alfalfa was cut 3 times in 1955 and 1956. It was cut only once in 1952 because of a heavy infestation of leaf hopper after the first cutting and only twice in 1954 because of poor regrowth due to dry weather following the second har-

TABLE 1.—*Summary of stands of alfalfa and red clover from Arasan-treated and untreated seed, Lafayette, Indiana, 1952-1956*

Crop and year	No. of seed lots in test	Average percentage stand[a]		No. of lots with statistically significant increases from treatment
		Arasan	Untreated	
Alfalfa				
1952	3	39	36	0
1953	3	48	46	0
1954	6	31	31	0
1955	22	35**	30	3
1956	30	66**	59	6
Total	64			9
Average		44	40	
Red clover				
1952	3	45	47	0
1953	2	53	50	0
1954	4	25	24	0
1955	22	30	30	0
1956	30	65	62	3
Total	61			3
Average		44	43	

[a] Average of 10 replications of 200 seeds for each lot. Double asterisks indicate statistical significance at the 1 per cent level.

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TABLE 2.—*Seedling stand from Arasan-treated and untreated alfalfa and red clover seed lots of different germination, Lafayette, Indiana, 1952-1956*

Crop and germination percentage	No. of seed lots in test	Average percentage stand [a]		No. of lots with statistically significant increases from treatment
		Arasan	Untreated	
Alfalfa				
Above 90	22	57	51	4
80–89	15	55	47	3
70–79	9	42	38	0
Below 70	18	42	37	2
Red Clover				
Above 90	9	47	47	0
80–89	24	60	57	2
70–79	17	43	43	0
Below 70	11	31	30	1

[a] Average of 10 replications of 200 seeds for each lot.

vest. The forage yield data were converted to tons per acre at 12 per cent moisture content. The stand and yield data were analyzed statistically and differences that were statistically significant at the 5 or 1 per cent level are referred to as significant.

RESULTS.—Table 1 gives a summary of the seedling stands of alfalfa and red clover from Arasan-treated and untreated seed. In only 2 of the 5 years, 1955 and 1956, was the average percentage stand from all lots of treated alfalfa seed significantly greater than that from untreated seed. The average percentage stand from treated red clover seed was not significantly greater than that from untreated seed in any of the 5 years.

Stands from 9 of the 64 individual lots of alfalfa tested, or 14 per cent, were significantly greater from treated seed than those from untreated seed. Only 3 of the 61 lots of red clover or 5 per cent of the lots tested showed a significantly greater stand from treated than from untreated seed. None of the seedling stands of alfalfa or red clover from treated seed were significantly smaller than those from untreated seed.

The responses to seed treatment of seed lots of different germination are presented in Table 2. As would be expected, the average percentage stand was greater in each successively higher germination group, with the exception of the red clover that germinated above 90 per cent. This inconsistency was attributable to the relatively few lots in this group in 1956, when stands were the highest. The differences between the average stand from treated and that from untreated seed for the lots above 80 per cent germination were as great as or greater than treatment differences of lots below 80 per cent germination. Furthermore, 7 of the 9 lots of alfalfa and 2 of the 3 lots of red clover that showed a significantly greater stand from treatment were above 80 per cent germination.

There were no significant differences between yields from treated and untreated alfalfa seed in any of the 4 years (Table 3). The only significant differences between yields of treated and untreated red clover seed were the lower yields from treated seed in 1952 (Table 4).

The red clover yields were significantly lower at the seeding rates of 2 and 4 lb. per acre in 1952 than at the higher seeding rates, but these were the only significant differences in yield between rates of planting. The highest yields of both alfalfa and red clover generally

TABLE 3.—*Forage yield of alfalfa grown from treated and untreated seed planted at various rates, Lafayette, Indiana, 1952-1956*

Year and treatment	Tons per acre at indicated seeding rate [a]						Mean for treatment
	2 lb.	4 lb.	6 lb.	8 lb.	10 lb.	12 lb.	
1952 [b]							
Arasan	1.08	1.52	1.38	1.57	1.34		1.38
Vancide 51	1.15	1.15	1.60	1.38	1.48		1.35
Untreated	1.22	1.56	1.53	1.45	1.81		1.47
Mean for rate	1.15	1.41	1.51	1.47	1.48		
1953 [c]							
Arasan			4.03	4.37	4.38	4.20	4.25
Vancide 51			4.44	4.11	4.29	4.21	4.26
Untreated			4.02	4.03	4.27	4.06	4.10
Mean for rate			4.16	4.17	4.31	4.16	
1955 [d]							
Arasan			6.77	6.53	6.88	6.85	6.76
Vancide 51			6.17	6.46	7.41	7.19	6.81
Untreated			6.36	6.62	6.87	6.81	6.67
Mean for rate			6.43	6.54	7.05	6.95	
1956 [d]							
Arasan			6.39	6.04	6.72	6.40	6.39
Vancide 51			6.36	5.59	6.82	6.78	6.39
Untreated			6.37	5.81	6.31	6.48	6.24
Mean for rate			6.37	5.81	6.62	6.55	

[a] Average of 4 replications planted in 5-row plots with the rows 7 in. apart and 25 ft. long. Differences not statistically significant.
[b] One cutting.
[c] Two cuttings.
[d] Three cuttings.

TABLE 4.—*Forage yield of red clover grown from treated and untreated seed planted at various rates, Lafayette, Indiana, 1952-1956*

Year and treatment	Tons per acre at indicated seeding rate[a]						Mean for treatment[b]
	2 lb.	4 lb.	6 lb.	8 lb.	10 lb.	12 lb.	
1952							
Arasan	1.41	2.20	2.79	3.49	2.93		2.56
Vancide 51	1.54	1.62	2.71	2.82	3.04		2.35
Untreated	1.81	2.04	3.22	3.37	3.58		2.80
Mean for rate[c]	1.59	1.95	2.91	3.23	3.18		
1954							
Arasan			3.95	3.91	4.25	4.32	4.11
Vancide 51			4.15	4.09	4.16	4.12	4.13
Untreated			4.05	4.17	4.21	4.26	4.17
Mean for rate			4.05	4.06	4.21	4.23	
1955							
Arasan			6.57	6.57	6.86	6.84	6.71
Vancide 51			6.37	6.79	6.99	6.72	6.72
Untreated			6.35	6.61	6.94	6.91	6.70
Mean for rate			6.43	6.66	6.93	6.82	
1956							
Arasan			5.84	6.20	6.37	6.47	6.22
Vancide 51			6.00	6.17	6.39	6.46	6.26
Untreated			6.09	6.38	6.14	6.43	6.26
Mean for rate			5.98	6.25	6.30	6.45	

[a] Average of 2 cuttings from 4 replications planted in 5-row plots with rows 7 in. apart and 25 ft. long.
[b] LSD for 1952 is 0.23 at the 5 per cent level and 0.32 at the 1 per cent level.
[c] LSD is 0.78 at the 5 per cent level and 1.09 at the 1 per cent level.

were obtained at the 10- and 12-lb. rates of seeding; however, there was no indication that the differences in yield between treated seed and untreated seed were any greater at the lower seeding rates, where small differences in stand might be expected to affect yield more markedly than at the higher rates.

DISCUSSION.—The results of these studies are in general agreement with those conducted elsewhere. Increases in average stand from Arasan-treated alfalfa of 1.3, 5.8, and 3.6 per cent and from Arasan-treated red clover of 3.3, 2.6, and 1.1 per cent were reported by Kreitlow et al (7), Kernkamp (6), and the writer, respectively.

Allison and Torrie (1), Gerdemann (4), Hanson (5), and Tyler et al (8) reported no statistically significant increases in seedling stand from seed treatment; however, Allison and Torrie (1), Buchholtz (3), Kreitlow (7), Kernkamp (6), Gerdemann (4), and Hanson (5) demonstrated that seed treatment did greatly improve the stand in Pythium-infested soil and in soils containing damping-off organisms. With the exception of the limited test reported by Brentzel (2), the other published reports of increased stands from seed treatment have been from greenhouse tests.

Kernkamp (6) reported substantial increases in seedling stands from mechanically injured alfalfa and sweet clover seed treated with Arasan. Mechanically damaged or lower germinating seed of other crops are known to be benefited most by seed treatment. The results obtained in this investigation did not indicate that the lower germinating lots of alfalfa and red clover benefited any more than did the higher germinating lots.

The reason for treating seed of any crop is to obtain good stands of vigorous, healthy plants that will produce increased yields. The results presented here help substantiate previous reports and do not justify a general recommendation for alfalfa and red clover seed treatment. The fact that seed treatment will increase the stand under specific conditions makes it a practice to be considered when difficulty has been met in obtaining a satisfactory stand of seedlings.

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MUTATION FOR INCREASED PATHOGENICITY IN PUCCINIA GRAMINIS VAR. TRITICI[1]

I. A. Watson[2]

SUMMARY

Greenhouse studies were made to determine if spontaneous mutations for pathogenicity in *Puccinia graminis* var. *tritici* could be found. Inoculum of cultures derived from single urediospores of 4 races that differed in color and/or pathogenicity was increased on Little Club wheat. From inoculation of seedlings of Lee and Bowie wheat, 1 gray-brown and 2 orange mutants were obtained, each more virulent on Lee than was the parent. The mutant cultures were indistinguishable from their respective parents on any varieties of wheat except Lee, Gabo, Timstein, Gaza, and Kenya C6042. No genetic change in pathogenicity on Bowie was observed in any of the cultures. Lee is considered a variety especially suitable for detection of mutants whereever Bowie appears to be unsuitable.

The mode of origin of new races of the wheat stem rust fungus, apart from those presumed to have arisen on barberry, has received little attention in North America. The origin of new races or biotypes by means of asexual processes is most important in areas where barberry seldom if ever rusts.

Recently, several reports (4, 5, 6) have appeared in the literature to show that nuclear exchange can occur in the dicaryophase, although it is still unknown whether this phenomenon has been responsible for the production of races now important. Mutation also is believed to be significant (3), but mutants have not yet been produced at will in the laboratory.

The present paper reports experiments in which spontaneous changes in pathogenicity were observed in *Puccinia graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Guyot in the greenhouse.

REVIEW OF LITERATURE.—The subject of mutation in *P. graminis* var. *tritici* has been reviewed by Johnson and Newton (3). In Australia, Waterhouse (8) made surveys of physiologic races of *P. graminis* var. *tritici* over a 30-year period and concluded that a single race made up about 90 per cent of the inoculum prior to the release of the variety Eureka. Eureka was resistant to this race and, so far as was known, to all other races present. With the cultivation of Eureka, a new biotype of the prevalent race was isolated; it differed from the prevalent race only in its ability to attack Eureka and all varieties genetically related to it. When Eureka was replaced by varieties having a different source of resistance, new races of the rust fungus were isolated from them. Waterhouse (8) has suggested this as being due to a continuing process of mutation, since greenhouse and field studies had not shown these races to have been present previously and since all appear to be related. Watson (10) used a single culture of race 222 Anz 3[3] to induce an epidemic of stem rust in the field; pustules yielding a new race, 222 Anz 4, were isolated from hybrid material of the cross ((Illinois-1 × Chinese)[2] × *Trticum timopheevi* (C.I. 12632)) × Eureka, which was resistant to race 222 Anz 3. This isolate was distinguishable from race 222 Anz 3 only by its reaction on the seedlings of variety C.I. 12632.[4] This new isolate was interpreted as a further example of mutation for increased pathogenicity.

Watson and Singh (11) have suggested that the increase in prevalence of the races with the wider host range has been due to the screening effects of the varieties cultivated rather than to more aggressiveness on the part of the pathogen.

MATERIALS AND METHODS.—In laboratory work with races of *P. graminis* var. *tritici* with red spores, marked changes in pathogenicity have been attributed to mutation on the assumption that less spectacular changes could be due to heretofore unobserved contamination. To offset this latter difficulty, races of the rust fungus were selected that were unusual for spore color and/or pathogenicity so that any mutants arising could be traced back readily to the parental type. The 4 races chosen were as follows: 1) Race NR-1 (orange)—a new race obtained from R. R. Nelson and characterized by its inability to attack Acme (on the regular host differential series, the rust reactions do not fit those of any of the classified races (7); 2) Race 11 (orange)—this culture, apart from color and pathogenicity, is characterized by the early production of telia on leaves of seedlings; 3) Race GB-1 (gray brown)—a culture obtained from barberries in 1955 and producing reactions on the regular differential series that do not correspond to those of any of the numbered races (see Table 1); 4) Race 111 (red)—a culture also obtained from barberries (it has never been collected on wheat in the field) and characterized by the fact that Little Club is the only variety of the regular differential series on which this isolate was pathogenic.

Four typical single-spore cultures, one from each race, were increased separately on Little Club until approximately 1000 plants of this variety were heavily rusted. This inoculum from each culture was then brushed onto an additional 1000 plants of Little Club and onto about 500 of Lee. The procedure was so ar-

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[3] The suffix Anz indicates that this is a biotype found in the Australia-New Zealand geographical area and differentiated by varieties found useful there.

[4] C.I. refers to accession number of Cereal Crops Section, U. S. Department of Agriculture.

TABLE 1.—*Reaction*[a] *of 19 wheat varieties to 4 parental races of Puccinia graminis var. tritici and mutants derived from 3 of them*

Variety	Race 111 (red)	Parent Race NR-1 (orange)	Mutant Race NR-2 (orange)	Parent Race 11 (orange)	Mutant Race 11-1 (orange)	Parent Race GB-1 (gray brown)	Mutant Race GB-2 (gray brown)
Little Club	3+	3+	3+	3+	3+	3+	3+
Marquis	0;	3+	3+	3+	3+	2	2
Reliance	0;	3+	3+	3+	3+	0	0
Kota	0;	3	3	3+	3+	3	3
Arnautka	0;	0;	0;	3+	3+	0;	0;
Mindum	0;	0;	0;	3+	3+	0;	0;
Spelmar	0;	0;	0;	3+	3+	0;	0;
Kubanka	0;	X	X	3+	3+	3+	3+
Acme	0;	0;	0;	3+	3+	3+	3+
Einkorn	0;	3+	3+	3+	3+	0;	0;
Vernal	0;	3+	3+	0;	0;	X	X
Khapli	0;	;1	;1	;1	;1	;1	;1
Lee	0;	;2=	X‡	;2=	X	;2=	2‡
Bowie	0;	;1	;1	;1	;1	1‡	1‡
Gabo	0;	;2=	2, 3	;2=	2, 3	;2=	2+, 3
Timstein	0;	;2=	2, 3	;2=	2, 3	;2=	2+, 3
(Ill.-1 × Chinese)[2] × *T. timopheevi* C.I. 12632	0;	X=	X=	3+	3+	3+	3+
Gaza	0;	0;	2	0	2	0;	2
Kenya C6042	0;	0;	2	0;	2	0;	2

[a] Infection types are as follows: 0—immune, ;—flecks, 1—very resistant, 2—moderately resistant, 3—moderately susceptible, 4—very susceptible, X—heterogeneous. Plus and minus signs are used to indicate variation within a given type: ‡ and = indicate the upper and lower limits, respectively, of each type.

ranged that new inoculations were made with each culture onto Little Club and Lee at intervals of 2–3 days. In each case, the resulting inoculum on Little Club was used to inoculate additional plants of Little Club and Lee. All inoculations were made on seedling plants and in a plastic-covered humidity chamber described by Hayden (2).

Race 111 (red) was treated differently since it is avirulent on all varieties except Little Club. After the initial increase, the inoculum was placed on about 1000 plants of Little Club; thereafter, at intervals of 2–3 days it was placed on Little Club, on approximately 100 plants of each of the varieties of the regular differential series, on 500 plants of Lee, and on 500 plants of Bowie.

EXPERIMENTAL RESULTS.—*With race NR-1 (orange).*—With this culture, seedlings of Lee gave the reaction type 0; to 2 =.[5] After the inoculum had been increased on approximately 10,000 plants of Little Club and used to inoculate 4000–4500 plants of Lee, 1 single orange pustule appeared on a Lee plant. The spores from this pustule were increased on Lee on which it produced infection type X‡. Inoculations were made separately from both the susceptible- and resistant-type pustules, and in each case the X‡ infection type was again produced on Lee.

Six single-spore cultures were made from the susceptible-type pustles, but the progeny of each also continued to produce the X‡ infection type on Lee. One of these mutants was carried in bulk through 16 uredial generations, and no change in virulence was observed.

It was impossible to distinguish between race NR-1 and the mutant (race NR-2) on the regular differential varieties either by color or by pathogenicity; however, they were clearly distinguishable on the varieties Lee, Gabo, Timstein, Gaza, and Kenya C6042. No other variety was found useful for their differentiation.

With race 11 (orange).—The mutant, race 11-1, arose from a single-spore culture of race 11 (orange) after approximately 6000 plants of Lee had been inoculated. It became evident first as a single susceptible-type pustule on Lee; when spores were increased from this pustule, they produced an X type infection on Lee. This mutant was apparently less virulent on Lee than was race NR-2. Single-spore cultures demonstrated that the mutant was not a mixture of races. Moreover, it has been maintained through 16 uredial generations without loss or change of virulence. Throughout this period it also had the tendency to form telia readily on seedlings, especially at higher temperatures; this character apparently has been inherited from the parental race 11 (orange). As with race NR-2, the only varieties suitable for distinguishing between the orange parent (race 11) and the mutant, race 11-1, were varieties Lee, Gabo, Timstein, Gaza, and Kenya C6042.

With race GB-1 (gray brown).—A gray-brown mutant appeared on Lee after approximately 2000 plants of Lee had been inoculated with race GB-1. This mutant is characterized by a 2‡ reaction type on Lee. As shown in Table 1, the varieties related to Lee and to Kenya C6042 are suitable for distinguishing between

[5] Infection types are as follows: 0—immune, ;—flecks, 1—very resistant, 2—moderately resistant, 3—moderately susceptible, 4—very susceptible, X—heterogeneous. Plus and minus signs are used to indicate variation within a given type: ‡ and = indicate the upper and lower limits, respectively, of each type.

the mutant and the parent.

With race 111 (red).—The reaction of all varieties of the regular differential series, except Little Club, to race 111 was 0; when greenhouse temperature averaged between 70° and 75°F. The reaction was 3° on Little Club. Inoculum was produced on approximately 20,000 plants of Little Club and was used to inoculate at different times a total of at least 600 plants of each of the varieties of the regular differential series. In addition to these, at least 6000 plants of Lee and 6000 of Bowie were inoculated with this race. No change in virulence was observed on any of these varieties.

With the mutants from race NR-1 and from race 11 (orange) on Bowie.—Single-spore cultures of these mutants were increased on Little Club and then tested for possible increased pathogenicity as previously described to see if cultures now virulent on Lee would mutate for increased virulence on Bowie. Bowie was resistant to both the parental and the mutant cultures of these 2 races (Table 1). Also, inocula were collected from at least 20,000 plants of Little Club inoculated with the mutant race NR-2 and from 20,000 inoculated with the other mutant, race 11-1. Each inoculum was used to inoculate more than 5000 plants of Bowie. No isolate was found that was more pathogenic on Bowie than was the original culture.

DISCUSSION AND CONCLUSIONS.—The results obtained in these experiments indicate that changes in pathogenicity may occur spontaneously in *P. graminis* var. *tritici* and that the resulting mutants can be readily isolated provided the screening variety is homogeneous and possesses genes suitable for separating the mutant from the parental culture. With the techniques and material used, it was impossible to calculate the mutation rate in these races. Moreover, it is impossible to say whether the changes have been gene mutations or changes involving 2 or more genes. The mutations reported from Australia, however, have been interpreted as stepwise mutations for virulence (9, 10, 11) involving 1 gene in the host at each step and paralleling the stepwise selection for resistance that has been practiced in the breeding program. This is in conformity with the gene-for-gene hypothesis (1), although the data are still inadequate to show that this is true in *P. graminis* var. *tritici*. The results from the present work are in agreement with this interpretation.

Although mutants were readily found on Lee in 3 of the 4 cultures used in this investigation, in no case was a mutant more virulent on Bowie than were its parental cultures. It is possible that this is connected with differences in factors for resistance carried by Bowie and Lee. Gabo (and presumably Lee) has 2 linked dominant complementary factors for resistance to the parental orange cultures, and apparently this system is less effective in protecting the plant against mutants than is the one possessed by Bowie. It is significant, however, that in both cultures a number of susceptible-type pustules were evident on certain plants of Bowie and that these appeared to distinguish clearly between 2 types of the rust fungus. Lee appears to be a variety in which the majority of the plants are suitable for selecting mutants, whereas the great majority of Bowie plants apparently were unsuitable for this purpose.

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FURTHER STUDIES ON THE PRODUCTION OF NEW RACES FROM MIXTURES OF RACES OF PUCCINIA GRAMINIS VAR. TRITICI ON WHEAT SEEDLINGS[1]

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SUMMARY

Cultures were obtained from single urediospores of 2 races of *Puccinia graminis* var. *tritici*. One had red spores and was weakly pathogenic on 14 wheat varieties, whereas the other had orange spores and was virulent on 8 of these same varieties. When a spore mixture of the 2 isolates (in equal proportions) was used to inoculate seedlings of the regular differential varieties, 4 virulent red races arose. The avirulence on Acme, a character shown by the parent races, was transmitted to 2 of the red virulent races recovered. Virulence or avirulence on Acme was associated with a similar reaction on Arnautka, Mindum, and Spelmar. The results are interpreted as showing further evidence of genetic variation in the dicaryophase of *P. graminis* var. *tritici*.

From the work already reported (4, 5), it has been suggested that nuclear exchange occurs in the dicaryophase of *Puccinia graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Guyot. Since the result of an exchange is observed so infrequently, it would appear that new dicaryons arising in this way either do not express themselves readily or that actual exchange of nuclei is a rare phenomenon. Fusion bodies of the type recorded by Rodenhiser and Hurd-Karrer (6) occur commonly under artificial conditions, but it is still not known whether such structures provide passage for nuclei. It would appear from previous work that races differ in their tendency to exchange nuclei or to form heterocaryons, but no cases have been found where new races are recurrently isolated as a result of mixing 2 or more single cultures on wheat plants. Dicaryons most likely to exchange nuclei would appear to be those arising from wide crosses such as intervarietal crosses of *P. graminis*.

Cereal rusts can seldom be cultured without the possibility of some contamination, unless special precautions are taken. Therefore, in order to gather conclusive data on nuclear exchange between vegetative hyphae of cultures of the rust fungus, it is essential to work with races that carry distinct marker characters. This paper reports the recovery of new virulent races of *P. graminis* var. *tritici* that were derived by mixing, on wheat seedlings, urediospores of an avirulent red race and a virulent orange race.

MATERIALS AND METHODS.—Two races of *P. graminis* var. *tritici* were used; one, race 111, had red urediospores and was virulent and sporulated on only Little Club wheat of the differential varieties, whereas the other, race NR-2, had orange urediospores and was avirulent on Acme (9).

Cultures derived from single urediospores of red race 111 and of orange race NR-2 were tested for at least 8 uredial generations on varieties of the standard differential series; both produced consistent reactions (Table 1). Inoculum of each race was increased on Little Club; approximately 1 g of dry spores of each race was collected and the 2 kinds thoroughly mixed together. Approximately 2 g of the spore mixture was dusted on leaves of 100 seedlings of each of the standard differential varieties plus Bowie and Lee. The plants were then incubated for 24 hours in a humidity chamber similar to that used by Hayden (1). The inoculated seedlings were observed after 10 days for the presence of red pustules. Those that appeared were isolated and identified in the usual way (8). All tests were repeated 3 times.

EXPERIMENT RESULTS.—Ten days after the plants were inoculated with the spore mixture, red pustules appeared on Marquis, Reliance, Vernal, and Einkorn. Because of the heavy concentration of inoculum, many pustules coalesced and it was not always possible to distinguish between resistant and susceptible infection types. Pustules producing red spores were densely crowded by those producing orange spores. Therefore, seedlings of Little Club were inoculated with red spores from the 4 varieties mentioned above. The inoculum was applied sparingly to insure development of scattered pustules; urediospores from these pustules were then identified on differential varieties.

Although red pustules occurred on Einkorn, only red race 111 was reisolated. Because this race sporulated on Einkorn when grown in association with race NR-2, a synergistic effect was apparent since pustules of red race 111 have not been found previously on this variety. Red race 111 also was recovered from Marquis, Reliance, and Vernal, but these isolations yielded new red races as well.

The parental races were recovered from all 3 trials; however, in the first trial, 3 new red races also were recovered, viz. races 34, NR-3, and NR-4. Their reactions on the differential varieties are shown in Table 1. The reactions of races NR-3 and NR-4 are different from any of those previously described by Stakman et al (8).

Isolation of urediospores from red pustules in the second trial yielded races 196 and NR-3, whereas races 34 and 196 were obtained from red pustules in the third test. Isolations from all orange pustules yielded only race NR-2. The reactions of 4 red races

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TABLE 1.—*Reactions of 15 wheat varieties to parental and derived races of Puccinia graminis var. tritici*

Variety	C.I. No. [a]	Reaction [b] to indicated parent race		Reaction [b] to indicated new red combination			
		Race 111 (red)	Race NR-2 (orange)	Race NR-4	Race 196	Race NR-3	Race 34
Little Club	4066	4	4	4	4	4	4
Marquis	3641	0;	3+	3+	3+	3+	3+
Reliance	7370	0;	3+	0	3+	0	3+
Kota	5878	0;	3	3	3	3	3
Arnautka	1493	0;	0;	0;	0;	3+	3+
Mindum	5296	0;	0;	0;	0;	3+	3+
Spelmar	6236	0;	0;	0;	0;	3+	3+
Kubanka	2094	0;	X	3+	3+	3+	3+
Acme	5284	0;	0;	0;	0;	3+	3+
Einkorn	2433	0;	3+	0;	0;	0;	0;
Vernal	3686	0;	3+	3+	0;	X+	0;
Khapli	4013	0;	;1	;1	;1	;1	;1
Lee	12488	0;	X‡	;1	;1	;1	;1
Bowie	13146	0;	0;	2=	2=	2=	2=
C.I. 12633 [c]		0;	X=	X=	X=	X=	X=

[a] C.I. refers to accession number of Cereal Crops Section, U. S. Department of Agriculture.

[b] Infection types are as follows: 0 = immune, ; = flecks, 1 = very resistant, 2 = moderately resistant, 3 = moderately susceptible, 4 = very susceptible, X = heterogeneous. Plus and minus are used to indicate variation within a given type: ‡ and = indicate the upper and lower limits, respectively, of each type (8).

[c] (Ill.-1 × Chinese)^2 × *Triticum timopheevi*.

identified from mixtures containing these 2 parental races are shown in Table 1. In the third test, the red spores from Marquis and Reliance were inoculated onto Little Club in such a way that single well-isolated pustules could be used to identify the race producing them. Of 37 pustules examined, 3 were caused by race 34, 6 by race 196, and 28 by the parental race, 111.

The 4 derived races have been maintained through 6 uredial generations and have given consistant reactions on differential varieties. Immature urediospores of the parental cultures and of the new red cultures were typically dicaryotic.

DISCUSSION AND CONCLUSIONS.—Precise information on the origin of the parental races used in this work is not available. Red race 111 was collected from barberry and its parents are unknown, whereas race NR-2 is probably a mutant from race NR-1.[3] Perhaps 1 or both of these parental races came initially from an intervarietal cross between *Puccinia graminis* Pers. var. *secalis* (Eriks. & E. Henn.) Seym. and *P. graminis* var. *tritici*. The new races are unusual in that each is avirulent on Acme. Such races are seldom found in North America; of the 189 races listed by Stakman and coworkers (8), only 23 are of this type. Johnson (3), in extensive selfing studies of *P. graminis* var. *tritici*, did not recover any races to which Acme was resistant; however, races avirulent on Acme occur commonly in crosses between *P. graminis* var. *tritici* and *P. graminis* var. *secalis* (2, 7).

Since avirulence on Acme is relatively rare, it is rather significant that at least 1 new red race to which Acme is resistant was recovered in each test involving a mixture. Of the 4 new red races recovered, 2 are avirulent on Acme and 2 are virulent (Table 1). Within each pair, further separation can be made by the reciprocal reactions on Reliance and Vernal.

The 2 races avirulent on Acme could be expected on the basis of the reaction of this variety to the parental races. It is not so simple to account for the other 2 races that were virulent on Arnautka, Mindum, and Spelmar, as well as on Acme. According to Johnson and coworkers (3), the virulence on Arnautka, Mindum, and Spelmar is inherited as a dominant character. If this principle applies here, then from the 2 parental races, which presumably lack the dominant gene for virulence, races NR-3 and 34, which are virulent on these varieties, have arisen. From Johnson's (2) work, it appears that genes for virulence on Arnautka, Mindum, and Spelmar in *P. graminis* var. *tritici* are in some way suppressed when associated with genes from *P. graminis* var. *secalis* in dicaryons. If this same phenomenon occurs in these parent races, then virulence on the durum varieties could result when the effects of a similar association are removed following nuclear exchange. The movement of the nuclei from one type of cytoplasm to another also may be an influencing factor, as suggested by Johnson (2). It is possible that the 4 new red races may have arisen by some sexual process, similar to parasexualism, that has not yet been satisfactorily explained. If more than 2 stable dicaryons arise, it must be assumed that either the cytoplasm is important in pathogenicity or the nuclei have combined in more ways than would be expected with 2 sex types.

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A SIMPLE 24-HOUR SLIDE SPORE COLLECTOR[1]

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SUMMARY

An inexpensive volumetric air sampler that collects spores on glycerol-coated microscope slides appears to be an improvement over volumetric samplers that concentrate the spore deposit on relatively small areas. The collection area is equally distributed over 24 slides, each hour's sample covering 1 sq. in., and there is minimal loss between hourly samples. The air volume of the collector (4.8 liters/minute or 0.17 cu. ft./minute) is such as effectively to collect spores of very divergent sizes. Spore counts, for 24-hour periods, of 14 genera of fungi confirmed the reports of other investigators on the diurnal fluctuations in number of spores. In 1 group of genera, maximum spore concentrations occurred at night; in another, it occurred during the day.

Knowledge of the cycle of spore production and/or dissemination is necessary for adequate interpretation of disease development in the field. For example, the period of continued wetness necessary for infection and subsequent lesion formation by *Piricularia oryzae* Cav. has been shown by Andersen et al (1) and Hashioka (4) to be about 10 hours with temperatures of 68° to 73° F. Hashioka, using exposed slides placed in a rice field, has shown that with *P. oryzae* maximum spore dissemination occurs at night between 10 p.m. and 6 a.m. Under average conditions, it would therefore not be possible for spores disseminated during the night to produce infection that same night. Since many fungi have been found to have diurnal fluctuations in the number of spores occurring in the air, an inexpensive, accurate, easy-to-operate spore collector for field use in the study of various plant diseases is warranted.

The results of Hashioka (4) with spore dissemination of *P. oryzae* were verified by the authors using a number of spore collecting devices such as exposed glycerol-coated microscope slides, glycerol-coated microscope slides mounted on vane carriers, and motorized air samplers that drew air through cellulose acetate cloth filters. All of these devices proved unsatisfactory for various reasons. The writers, in attempting to develop an inexpensive collector that would produce an extensive collection area for observation, have devised the collector that is described below.

Description of the 24-hour slide spore collector.—A 24-hour suction-type collector (Fig. 1,A), which combined desirable features with simplicity of operation, was constructed. Air, at a rate of 4.8 liters/minute or 0.17 cu. ft./minute, was drawn through a ¼-in. copper tube such that it impinged on glycerol-coated microscope slides that were rotated past the orifice of the tube at the rate of 1⅛ in./hour, resulting in an hour's sampling area of 1 sq. in. of exposed microscope slide. Twenty-four slides were carried vertically on the periphery of a modified table of a Taylor dew meter (6). In this way, a complete day's sampling could be made without changing slides. Air flow was effected by means of a automobile defroster fan powered by a 6 volt-storage battery. This fan was mounted in a circular opening in the cover of the collector such as to force air out of the collection chamber, causing a negative pressure therein and an air flow through the copper tube in the collector wall. The defroster fan, slide holder, and copper tube with a slide situated in front of the orifice are shown in Figure 1 (A, B, and C, respectively). Direct microscopic examination of the slides may be made (Fig. 1,D).

Construction of the spore collector is relatively simple. The collector pictured in Figure 1,A was constructed from an old dynamite box, whereas that shown in Figure 1,E was made from welded sheet aluminum. The only requisite for the body of the collector is an airtight box with removable lid and inside dimensions

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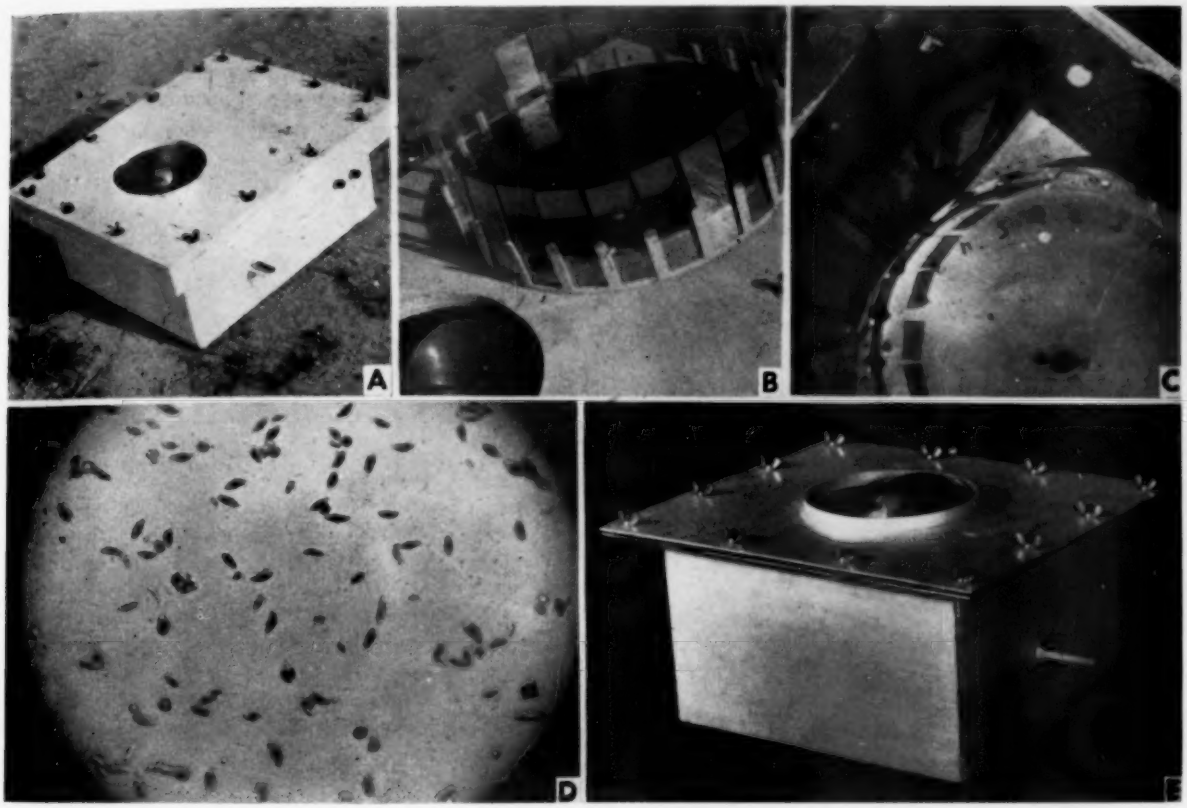

FIG. 1. The 24-hour slide spore collector. A) Over-all view. B) Slide holder. C) Air intake tube, with slide situated in front of the orifice. D) Typical microscope field (43×). E) Improved aluminum model.

of 12⅜ in. long, 10¾ in. wide, and 7¾ in. high. A hole is then made in the top of the box to allow the defroster fan to be mounted within this hole in such a manner that the blade of the fan is centered between the top and bottom surface of the lid and will force air out of the box. A 5-in. copper tube is then inserted into one of the small sides of the box in such a way as to be situated ⅛ in. from the horizontally mounted microscope slides. In the authors' collectors, the 24 microscope slides were mounted on the periphery of a modified table of a Taylor dew meter (6); however, any circular disc 8⅞ in. in diameter that can be mounted on a 24-hour clock is adequate. The slides can be held in a horizontal position by various means, although an easy method is to mount the plastic portion of Aloe-Sinclair Micro. Slide Holders (A. S. Aloe Co., St. Louis, Mo., Catalog No. 59260) around the periphery of the table by heating the holders, bending them to shape, and screwing or bolting them to the table.

Typical results obtained with the 24-hour slide spore collector.—Previous investigations on spore counts of the air by means of various unsatisfactory methods had indicated that the spores of some species were more prevalent in daytime collections than in those made during the night whereas the reverse was true for other species. In addition, it was noted that the spores of *P. oryzae* were most abundant in samples collected between 4 and 6 a.m. Gregory and Hirst (3) observed a diurnal fluctuation of the basidiospore content of the air, counts in the early morning hours running 3–5 times those made during the day. Thus collections were made by means of the 24-hour slide spore collector to see if similar diurnal fluctuations for spore content of the air and peaks for the spores of *P. oryzae* could be found. The terminology used by Gregory (2) will be used hereafter; the spores found in greatest abundance during the daytime will be referred to as "day spora" and those found at night as "night spora."

When mean spore concentrations of several typical 24-hour collection periods were considered, it was found that 7 genera (*Leptosphaeria*, *Epicoccum*, *Piricularia*, *Cladosporium*, *Diplodia*, *Ophiobolus*, and an unidentified genus causing mildew) fell into the night spora group; 6 genera (*Nigrospora*, *Penicillium*, *Alternaria*, *Curvularia*, *Tricoconis*, and an unidentified genus of a smut fungus) were far more abundant during the day than during the night. In general, the night spora were found between 6 p.m. and 9 a.m. and the day spora between 8 a.m. and 5 p.m.

Typical curves for fungus spores of all species, for a typical species of the day spora group, and for *P. oryzae*, a typical species of the night spora group, are presented in Figure 2 (A, B, and C, respectively).

Although the time of maximum concentration of

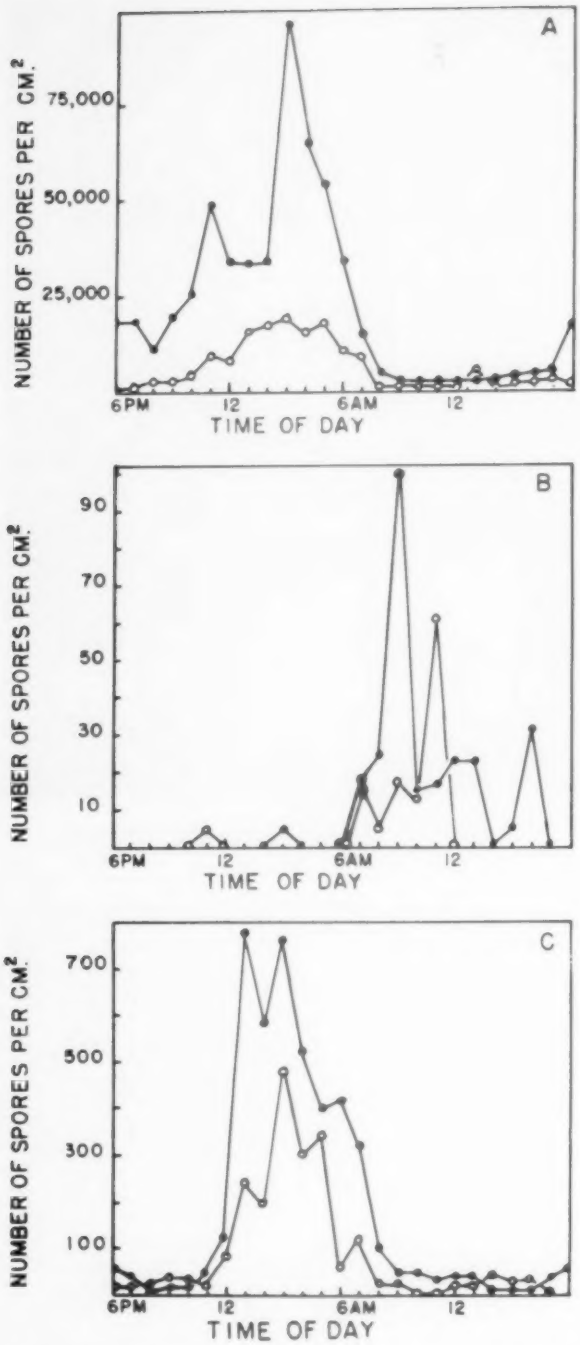

FIG. 2. Hourly spore concentrations of various fungi collected with the 24-hour slide spore collector. Open circles denote collection during a day with low spore counts, closed circles during a day of high spore counts. A) Spores of all species. B) *Alternaria* sp., typical of the day spora group. C) *Piricularia oryzae* Cav., typical of the night spora group.

spores of *P. oryzae* differed slightly from that found by the other methods of collection, the same trend was apparent; consequently, *P. oryzae* can safely be classified as belonging to the night spora group.

Even though spores of a few species were collected only during the day, the vast majority of spore material was found in the air during the night, maximum spore concentration occurring at approximately 3 a.m.

DISCUSSION.—A volumetric-type collector has been demonstrated by Hirst (5) to have a minimum efficiency of 45 per cent at a suction rate of 17.5 liters/minute and a minimum efficiency of 70 per cent at a lower suction rate of 10 liters/minute. Both efficiencies were computed with cross winds of 1–21 miles per hour blowing across the orifice of the collector. The volume of air used in the 24-hour slide spore collector is 4.8 liters of air/minute. Thus by analogy with the "Hirst" collector, the efficiency of the 24-hour slide spore collector should be 70 per cent or greater, since the suction rate of the collector is well below that of the "Hirst" machine when the latter is known to be operating at a minimum of 70 per cent.

Glycerol is recommended as a slide coating, for it preserves and hydrates the spores without distortion, making identification by direct microscopic examination easy. Other adhesive materials such as Vaseline and gummed tape have been used, but in our opinion all have some disadvantages.

The volumetric collector was effective over a wide range, from small spherical spores of about 5 μ diameter to the spores of *Helminthosporium oryzae* (*Cochliobolus miyabeanus* (Ito & Kuribay.) Drechs. ex Dast.), which attain lengths up to 140 μ.

The results obtained with this collector corroborate the findings of Hashioka (4) and the previous work of the writers with exposed slides, slides on vanes, etc. for spores of *P. oryzae;* also, they were in agreement with those results reported by Gregory (2) and Gregory and Hirst (3) with respect to night spora and day spora. It is efficient, simple to operate, and permits convenient observation of the collection area.

The cost of the instrument illustrated in Figure 1.A was approximately $15.00, plus the additional cost of a 6-volt automobile storage battery and a clock motor. The improved model (Fig. 1.E), with the case made of aluminum instead of wood, was developed at a cost of approximately $60.00, not including the battery.

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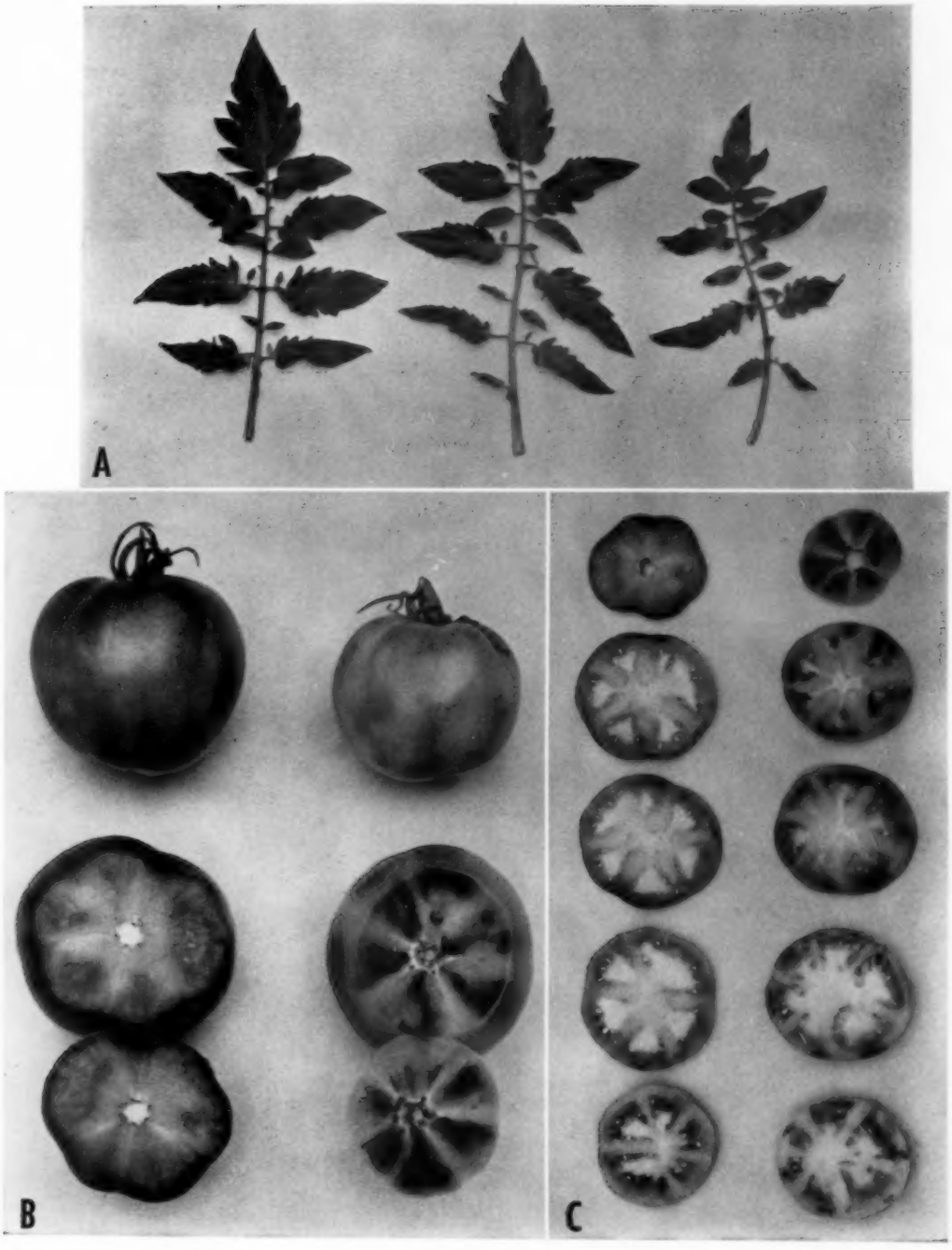
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The April 1957 issue of PHYTOPATHOLOGY, Volume 47, Number 4, contained a paper by John S. Boyle and David C. Wharton titled "The Experimental Reproduction of Tomato Internal Browning by Inoculation with Strains of Tobacco Mosaic Virus." The color plate included in this paper was, in some copies, defective in showing spots of red where no such spots were intended. The plate is reprinted here in its proper form. If the plate in your April issue is defective, please replace it with this correct print.